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UNDERSTANDING THE MOLECULAR MECHANISMS OF S-ADENOSYLMETHIONINE-ANTITUMOR ACTIVITY IN LARYNGEAL SQUAMOUS CANCER CELLS

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ABBREVIATIONS

AdoMet	S-Adenosyl-L-methionine
AdoHcy	S-Adenosyl-L-homocysteine
AGO	Argonaute protein
AMPK	AMP-activated protein kinase
Annexin V-FITC	Annexin V-fluorescein isothiocyanate
APEX1	DNA-(apurinic or apyrimidinic site)lyase
AUF1	AU-rich element RNA-binding protein 1
BBR	Berberine
BSA	Bovine serum albumin
CCL2	chemokine (C-C motif) ligand 2 (CCL2)
CCR2	C-C chemokine receptor type 2 (CCR2)
cDDP	Cisplatin
cFLIP	FLICE-like inhibitory protein
CHOP	CCAAT-enhancer-binding protein homologous protein
CLC	Cloroquine
dcAdoMet	S-adenosyl-(5')-3-methylthiopropylamine
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DGCR8	DiGeorge syndrome critical regione gene 8
DICER	dsRNA specific ribonuclease
DROSHA	Double-strand-RNA-specific ribonuclease
DUSP1	Dual specificity phosphatase 1
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal regulated kinase 1/2
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Genomic instability
GSH	Glutathione
GSK-3β	β-Glycogen synthase kinase 3

HCC	Hepatocellular carcinoma
Hcy	L-Homocysteine
HGF	Hepatocyte growth factor
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human papillomavirus
HuR	Human antigen R
IC	Inhibitory concentration
IKK	Inhibitor of nuclear factor-κB
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-6	Interleukin-6
JNK	C-Jun N-terminal kinase
LC3B	Microtubule-associated protein light chain 3B
LKB1	Liver kinase B1
LSCC	Laryngeal Squamous Cell Carcinoma
LTR	LysoTracker-red
MAPK	Mitogen activated protein kinase
MAT	Adenosylmethionine synthetase or methionine adenosyltransferase
MAT1A-KO	Mouse knockout for *MAT1A* gene
MFI	Median fluorescence intensities
MKK4	Mitogen-activated protein kinase kinase 4
MLKL	mixed lineage kinase domain like pseudokinase
MMP-2	Matrix metalloproteinase-2
miRISC	miRNA-associated RNA-induced silencing complex
miRNA	MicroRNA
mRNA	Messenger RNA
MT1-MMP	Membrane type 1 matrix metalloproteinase
MTA	5'-deoxy-5'-methylthioadenosine
MTHF	Methyltetrahydrofolate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFKB	Nuclear factor-κB
NSCLC	Non small cell lung cancer
ODC	Ornithine decarboxylase

ORF	Open reading frame
P38	Mitogen- activated protein kinase 11
PARP	Poly (ADP ribose)polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
Pri-miRNA	Primary transcript RNA
qRT-PCR	Quantitative real-time PCR
RBP	RNA binding proteins
RIP3.	Receptor-interacting protein kinase 3
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SAH	S-Adenosylhomocysteine
SAM or SAMe	S-Adenosyl-L-methionine
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SR protein	Serine/arginine-rich protein
STAT3	Signal transducer and activator of transcription 3
sXBP1	Spliced form of X-box binding protein 1
TBS	Tris-buffered saline
TRIS	Tris(hydroxymethyl)aminomethane
uPA	Urokinase-type plasminogen activator
UPR	Unfolded protein response
USP7	Ubiquitin carboxyl-terminal hydrolase 7
uXBP1	Unspliced form of X-box binding protein 1

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SOMMARIO

La S-adenosil-L-metionina (AdoMet o SAM) è un nucleoside solforato ubiquitariamente presente negli organismi viventi che esplica una varietà di funzioni biologiche ben documentate. Come è noto, infatti, il composto di solfonio rappresenta il principale donatore di gruppi metilici ed è anche il precursore della S-adenosilmetionina decarbossilata, il donatore di gruppi propilamminici nella biosintesi delle poliammine. La peculiare reattività dell'AdoMet è in gran parte legata alla presenza del polo di solfonio, che rende i tre atomi di carbonio legati allo zolfo siti elettrofili suscettibili di attacco nucleofilo, per cui la molecola è estremamente reattiva ed in grado di agire da donatore del gruppo metilico, del gruppo amminopropilico e del gruppo adenosilico. Nell'ultimo decennio molti studi sia *in vitro* che *in vivo* hanno evidenziato un coinvolgimento dell'AdoMet in diversi processi cellulari tra cui la proliferazione, il differenziamento, la regolazione del ciclo cellulare e l'apoptosi in diversi sistemi tumorali. Tuttavia, i meccanismi molecolari alla base dell'effetto esercitato dalla molecola non sono stati ancora del tutto chiariti.

Il mio lavoro di ricerca durante il corso di Dottorato è stato finalizzato allo studio sia del ruolo antitumorale esercitato dall'AdoMet in cellule di carcinoma della laringe, sia della regolazione dell'espressione dei microRNA in seguito al trattamento con il composto di solfonio. Al fine di valutare l'effetto antiproliferativo e i possibili meccanismi relativi all'attività antitumorale di questo importante composto fisiologico, è stata impiegata come sistema sperimentale la linea cellulare JHU-SCC-011 di tumore alla laringe, una linea cellulare molto ben caratterizzata e largamente utilizzata per ricerche biomediche *in vitro* e *in vivo* che rientra nella più ampia categoria dei tumori testa-collo.

I tumori della laringe rappresentano circa il 2-5% di tutte le neoplasie maligne dell'uomo e sono, assieme al cavo orale, le più frequenti della regione testa-collo. Nonostante la facile accessibilità del distretto testa-collo, la diagnosi di tali neoplasie è molto spesso tardiva e la relativa prognosi non è migliorata negli ultimi decenni. Frequentemente, vengono combinate per il trattamento di tale classe di neoplasie chirurgia, radioterapia e chemioterapia.

Il cisplatino (cDDP) è il principale farmaco usato per il trattamento di questa classe di tumori sebbene la sua applicazione clinica sia limitata dall'elevata tossicità e dallo sviluppo di meccanismi di resistenza. Dati gli effetti collaterali irreversibili legati alla somministrazione di questo chemioterapico, l'interesse della ricerca si è spostato all'individuazione di nuove molecole da associare in combinazione con il cDDP.

Inizialmente sono stati condotti esperimenti di vitalità cellulare in seguito a trattamento per differenti tempi con dosi crescenti di AdoMet (da 3 µM a 1mM). I risultati ottenuti hanno dimostrato che l'AdoMet esercita un effetto inibitorio sulla proliferazione cellulare in maniera dose e tempo dipendente, inducendo alla concentrazione di 300 µM una riduzione del 50% della vitalità cellulare dopo 72 ore.

Per valutare l'effetto biologico esercitato dall'AdoMet sono stati effettuati esperimenti di analisi dei principali meccanismi di morte cellulare tra i quali il processo apoptotico, il meccanismo autofagico, e il meccanismo di stress del reticolo endoplasmatico (ER-stress).

In particolare, l'analisi citofluorimetrica ha evidenziato che l'AdoMet alla dose di 300 µM è in grado di indurre il 4% e il 19% di apoptosi rispettivamente dopo 48 e72 ore dal trattamento. L'attivazione del processo apoptotico è stato anche confermato dall'aumento del rapporto pro-apoptotico Bax/Bcl2 e dalla riduzione nei livelli della pro-caspasi iniziatrice 9, delle pro-caspasi effettrici 6 e 7 e della poli (ADP ribosio) polimerasi (PARP), i cui livelli di espressione sono stati valutati mediante Western blotting.

L'analisi del processo autofagico è stata valutata mediante marcatura cellulare con lysotracker red, un composto che lega ed evidenzia le vescicole cellulari a pH acido, come le vescicole autofagolisosomiche. L'aumento dell'intensità di fluorescenza media nei campioni trattati alla concentrazione di 200 e 300 µM AdoMet dopo 24 e 48 ore è risultata maggiore rispetto ai controlli, fornendo così una prima indicazione che AdoMet è in grado di innescare l'attivazione del processo autofagico. Tale risultato è accompagnato dall'aumento dei livelli del principale marcatore autofagico "microtubule-associated protein light chain 3B" (LC3II).

Inoltre nella linea cellulare JHU-SCC-011 è stata valutata la capacità dell'AdoMet di indurre ER-stress. A tale scopo le cellule trattate con 200 e 300 µM AdoMet per 48 e 72 ore e marcate con ER-Tracker, un colorante vitale che si accumula nel reticolo endoplasmatico, sono state analizzate mediante microscopia a fluorescenza. L'AdoMet aumenta notevolmente la fluorescenza delle cellule JHU-SCC-011 rispetto alle cellule non trattate e causa un cambiamento morfologico del reticolo endoplasmatico, dato ascrivibile all'attivazione dell'ER-stress. L'attivazione dei marcatori coinvolti nel meccanismo di ER-stress sono stati valutati tramite qRT-PCR e Western blotting. I dati ottenuti mostravano un incremento trascrizionale dell'isoforma di splicing del gene *XBP1* (sXBP1) ed un aumento trascrizionale e traduzionale della proteina CHOP, principale mediatore del processo apoptotico attivato dall'ER-stress. Nella linea cellulare JHU-SCC-011, inoltre, è stato dimostrato che il trattamento con AdoMet induceva l'incremento del rapporto pERK/ERK, pJNK/JNK e

pP38/P38, proteine appartenenti alla famiglia delle proteine chinasi attivate dai mitogeni (MAPK). Uno studio approfondito della letteratura ha evidenziato una correlazione tra l'attivazione dell'apoptosi mediata dall'ER-stress e l'aumento dell'espressione delle MAPK, identificando l'ER-stress come il possibile meccanismo mediante il quale l'AdoMet innesca l'apoptosi.

L'attività anti-proliferativa e pro-apoptotica dell'AdoMet, unitamente al fatto che esso rappresenta un composto naturale la cui somministrazione non induce effetti collaterali, ci ha portati ad approfondire un possibile uso del composto di solfonio in sinergia con chemioterapici già impiegati per la terapia dei tumori testa-collo. È stato valutato, quindi, il possibile effetto sinergico dell'AdoMet con il cDDP. La combinazione delle due molecole è risultata fortemente sinergica, quando i due farmaci sono stati usati a concentrazioni di 200 µM AdoMet e 0,18 µM cDDP. L'analisi al FACS ha mostrato un incremento dell'apoptosi in seguito alla somministrazione dei due farmaci confermata dall'analisi mediante Western blotting che ha evidenziato un decremento evidente delle pro-caspasi 9 e 6 e della proteina PARP, ed un aumento del rapporto Bax/Bcl2, pERK/ERK1/2 e pJNK/JNK.

Complessivamente questi dati indicano che l'effetto pro-apoptotico dell'AdoMet potrebbe essere mediato dall'attivazione dell'ER-stress e che il suo utilizzo in combinazione con cDDP potrebbe indurre un miglioramento nell'approccio farmacologico al trattamento del carcinoma della laringe, associando all'efficacia terapeutica una minore tossicità del farmaco.

Recentemente una nuova classe di molecole di RNA non codificanti, noti come microRNA (miRNA), è stata associata a diverse malattie umane incluso il tumore della laringe.

Nella seconda parte della tesi è stato studiato il profilo di espressione dei miRNA in cellule JHU-SCC-011 in seguito al trattamento con 300 µM AdoMet per 72 ore, al fine di individuare il coinvolgimento diretto dei miRNA sull'inibizione della proliferazione cellulare esercitata dal composto di solfonio.

L'analisi effettuata mediante la tecnica dei microarray ha evidenziato un gruppo di miRNA sensibilmente regolati dall'AdoMet, quali: miR-187, miR-487b, miR-615-5p, miR-618 e miR-888-5p. Tra questi, il miR-888-5p è risultato significativamente down-regolato e tale dato è stato confermato mediante Real-Time PCR quantitativa (qRT-PCR). Per questo motivo si è scelto di proseguire gli studi con il miR-888-5p e trasfettare le cellule di carcinoma della laringe JHU-SCC-011 rispettivamente con miR-888-5p "mimic" ed "inhibitor", da soli, o con aggiunta di 300 µM AdoMet, per 72 ore. I miRNA "mimic" sono

piccole molecole di RNA a doppio filamento chimicamente modificate che mimano i miRNA endogeni e ne consentono l'analisi funzionale mediante l'up-regolazione dell'attività biologica. Viceversa, i miRNA "inhibitor" sono in grado di legare specificamente e inibire molecole endogene di microRNA e consentirne l'analisi funzionale mediante la riduzione dell'attività biologica. I dati ottenuti mostrano che il miR-888-5p inhibitor da solo è in grado di indurre l'attivazione del processo apoptotico, effetto ulteriormente potenziato dal trattamento combinato con l'AdoMet. Questi risultati sono stati confermati dall'analisi dei principali marcatori apoptotici, quali le pro-caspasi 9, 7 e 8 e della proteina PARP, i cui livelli di espressione risultano essere down-regolati dalla combinazione AdoMet e miRNA inhibitor.

Complessivamente questi dati contribuiscono alla comprensione del meccanismo d'azione esercitato dall'AdoMet nelle cellule di carcinoma alla laringe JHU-SCC-011, evidenziando il possibile coinvolgimento del miR-888-5p nell'inibizione dell'attivazione del processo apoptotico.

SUMMARY

S-Adenosyl-L-methionine (AdoMet, also known as SAM or SAMe), is an ubiquitous sulfur nucleoside exerting a variety of well-documented biological functions. It represents the main methyl donor required in numerous methylation reactions, and also the precursor of the decarboxylated S-adenosylmethionine, the propylamine group donor in polyamine biosynthesis.

The peculiar AdoMet reactivity is inherent to its sulfonium pole that makes the three carbon atoms (bound to the sulfur atom) highly susceptible to nucleophilic substitution; this renders the molecule able to donate the methyl group, the aminopropyl group and the adenosyl group.

In the last decade a lot of *in vivo* and *in vitro* studies highlighted the antiproliferative, anti-metastatic and pro-apoptotic effects of AdoMet in cancer cells. However, the molecular mechanisms underlying the effects exerted by the molecule have not yet been fully clarified.

My work, during the PhD, was aimed to the study of the antitumor activity exerted by AdoMet in laryngeal squamous cancer cells as well as at the regulation of microRNAs expression following treatment with the sulfonium compound. To evaluate the antiproliferative effect exerted by AdoMet and the molecular mechanism underlying the antitumor activity of this important physiological compound we used JHU-SCC-011, a cell line that is very well characterized and widely used for biomedical research *in vitro* and *in vivo*.

Carcinoma of larynx represents 2-5% of all tumors and is, together with oral cancer, one of the most frequent tumors in the head and neck region. Despite the easy accessibility of the head-neck district, the diagnosis of these neoplasms is very often late and the relative prognosis has not improved in recent decades. The treatment of these tumors include one or combination of the following: surgery, radiation, chemotherapeutic.

Cisplatin or cisplatinum (cDDP) is the major drug used to treat this class of tumor. However, the clinical application of cDDP is limited by the high toxicity and resistance mechanisms in cancer cells. To overcome this problem, a new line of research is aimed to identify new molecules and natural compounds to be used in combination with cDDP.

Firstly, to assay the cell viability we evaluated the anti-proliferative effects of AdoMet on JHU-SCC-011 cells after the treatment with increasing concentrations of AdoMet (ranging from 3 to 1mM) for different time. AdoMet causes a dose- and time-dependent reduction of

cell viability with an IC50 (50% inhibitory concentration) value of 300 μM, after 72 hours treatment.

To further explore the biological effects of AdoMet, we analyzed the principal death mechanism as apoptotic, autophagic and endoplasmic reticulum stress (ER-stress) processes by different assay.

In particular, FACS analysis highlighted the ability of sulfonium compound to induce apoptotic death in laryngeal squamous cancer cells. JHU-SCC-011 reached about 4% and 19% of apoptosis after 48 and 72 hours treatment with 300 μM AdoMet. The activation of apoptotic process was confirmed by a significant increase of Bax/Bcl-2 pro-apoptotic ratio, paralleled by poly (ADP ribose) polymerase (PARP) and caspase-9, 6 and 7 cleavages, as revealed by Western Blotting analysis.

The autophagy occurrence was evaluated by lysotracker red staining, a fluorescent probe for labeling and tracking of acidic organelles, including autophagosome and autolysosome structures. The results obtained revealed an increase in the average fluorescence intensity in cells treated with 200 and 300 μM AdoMet after 24 and 48 hours, compared to controls, providing a first indication that AdoMet was able to trigger autophagy process. This result was accompanied by the increase of the levels of the principal autophagic marker such as the “microtubule-associated protein light chain 3B” (LC3B-II).

Moreover, we have evaluated the ability of sulfonium compound to induce ER-stress in JHU-SCC-011cell line. Cells were treated with 200 and 300 μM AdoMet for 24 and 48 hours and analyzed by fluorescence microscopy using ER-Tracker, a vital dye that accumulates into ER. AdoMet greatly increased fluorescence of cells compared to untreated cells, and caused morphological changes of the ER, that appeared enlarged, due to the activation of the ER process.

To validate the effectiveness of AdoMet as an ER-stress inducer, we evaluated the activation of ER-stress markers, and their downstream signaling molecules by qRT-PCR and Western blotting. The quantitative analysis showed the increase of transcriptional sXBP1 levels and an increase in the transcriptional and translational levels of CHOP, the main mediator of the apoptotic process activated by ER-stress. Furthermore, AdoMet caused an increase of both pJNK/JNK, pERK/ERK1/2 and pP38/P38 ratio, proteins belonging to the family of protein kinases activated by mitogens (MAPK). A deep study of literature has shown a correlation between the activation of apoptosis by ER-stress and the increase in the

expression of MAPK, identifying the activation of ER-stress as a possible mechanism by which AdoMet triggers apoptosis.

The potential of AdoMet as antiproliferative and pro-apoptotic agent and the fact it is an approved nutritional supplement lead us to investigate its possible synergistic effects with other chemioterapics, used for the treatment of this kind of tumors.

To this purpose, we have used AdoMet in combination with cDDP. The drugs combination was found to be strongly synergistic, when the two drugs were used at concentrations of 200 μM AdoMet and 0.18 μM cDDP. The administration of the two drugs showed by FACS analysis an increase of apoptosis and by Western blotting a strong decrease of pro-caspase 9, pro-caspase 6, and PARP protein, and an increase of the Bax /Bcl2, pERK / ERK1/2 and pJNK / JNK ratio.

Overall, these data suggest that the activation of ER-stress represents a possible mechanism underlying the pro-apoptotic effect of AdoMet, and that the sulfonium compound can be used in combination with cDDP, or its analogs, in the treatment of laryngeal cancer, combining therapeutic efficacy with lower toxicity of the drug currently in use.

Recently, a new class of small non-coding RNA molecules, known as microRNAs (miRNAs), has been associated with several human diseases including laryngeal cancer.

In the second part of my thesis, it has been evaluated the regulation of miRNAs expression profiling following AdoMet-treatment in JHU-SCC-011 laryngeal cancer cell line, in order to detect the direct involvement of miRNAs on the inhibition of cell proliferation exerted by the sulfonium compound.

Microarray analysis after 72 hours of JHU-SCC-011 treatment with 300 μM AdoMet revealed different significantly modulated miRNAs like miR-187, miR-487b, miR-615-5p, miR-618 e miR-888-5p, compared to controls, all of them involved in the regulation of proliferation and/or survival. In particular, miRNA 888-5p was found to be significantly down-regulated and this result was then confirmed by quantitative real-time PCR (qRT-PCR).Afterwards, we transfected laryngeal cells with miR-888-5p mimic and inhibitor in free medium or medium supplemented with 300 μM AdoMet, for 72 hours.

The miRNA mimics are small molecules of chemically modified double stranded RNAs that mimic endogenous miRNAs and allow functional analysis by up-regulation of the molecule. On the other hand, miRNA inhibitors are able to specifically bind and inhibit endogenous microRNAs molecules and allow functional analysis by reducing biological activity.

The data obtained provide evidences that miR-888-5p alone induces apoptosis and this effect is enhanced by the addition of AdoMet. On the other hand, miR-888-5p mimic displays opposite effects. These results were confirmed by Western blot analysis of main apoptotic markers pro-caspase 9, pro-caspase 7, pro-caspase 8 and PARP proteins, whose protein levels were down-regulated by the AdoMet and miRNA inhibitor combination.

Overall, this study contributes to better understand the mechanism by which AdoMet exerts its antiproliferative and pro-apoptotic effect in laryngeal squamous cancer cells, highlighting the involvement of miR-888-5p in the inhibition of apoptotic process exerted by AdoMet.

INTRODUCTION

S-Adenosylmethionine

S-Adenosyl-L-methionine (AdoMet, also abbreviated with SAM or SAMe) is the principal biological methyl donor synthesized in all mammalian cells that plays a primary role in cellular metabolism since it is involved in a wide variety of important biochemical processes (1-4). The peculiar AdoMet biochemical as well as chemical properties are inherent to its sulfonium pole that makes the three carbon atoms (bound to the sulfur atom) highly susceptible to nucleophilic substitution; this renders the molecule extremely reactive and able to donate the methyl group, aminopropyl group and adenosyl group (1-2)(Fig.1).AdoMet and its decarboxylated product, S-adenosyl-(5')-3-methylthiopropylamine, represent the only sulfonium compounds detectable in mammalian tissues.

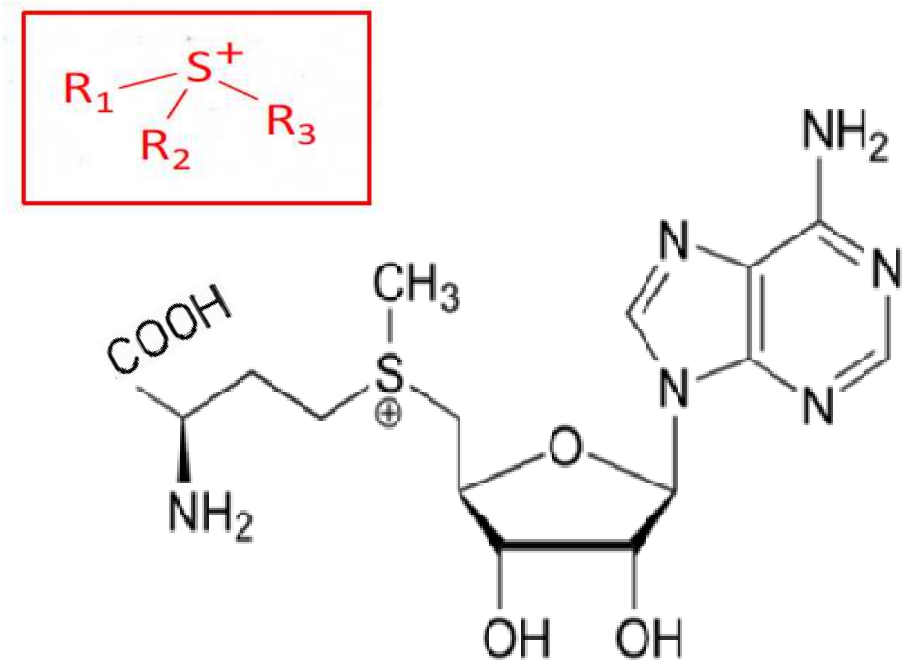

Fig. 1 - S-Adenosylmethionine and sulfonium ion (in red) chemical structure.

The central role of AdoMet in cellular metabolism is well known since its discovery by Giulio Cantoni, in 1952 (5).He elegantly demonstrated that AdoMet biosynthesis is performed starting from ATP and methionine by AdoMet synthetase or methionine adenosyltransferase (MAT) (EC 2.5.1.6), according to the reaction shown in Figure 2.

The reaction occurs in two-step mechanism, in which, firstly, the adenosyl group is transferred from ATP to the sulfur atom of methionine, leading to the synthesis of AdoMet and tripolyphosphate. In the second reaction, tripolyphosphate is hydrolyzed to orthophosphate and pyrophosphate allowing the release of the three products (6-8). All living cells, with the only exception of some parasites and infectious agents, which acquire AdoMet from their hosts, express MAT (9).

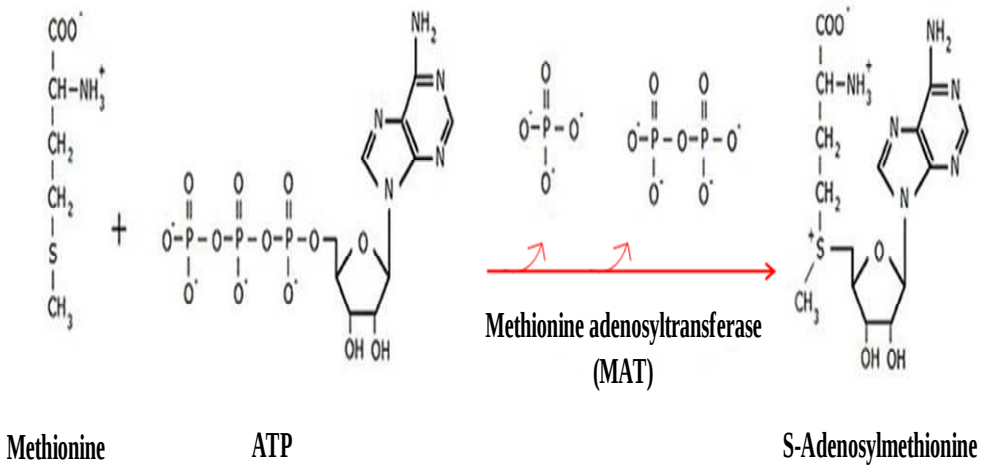

Fig. 2 - S-Adenosylmethionine biosynthesis.

MAT has been purified and characterized extensively in *Bacteria*, *Eukarya* and *Archaea* (10-14). Three distinct MAT genes, *MAT1A*, *MAT2A*, and *MAT2B* encode the protein products, MATα1, MATα2, and MATβ respectively. *MAT1A* is mainly expressed in liver (mostly hepatocytes), the major site of synthesis and utilization of AdoMet, and pancreatic acinar cells. It encodes for a 396 amino acid protein that forms wither a homodimer (MATIII) or a homotetramer (MATI). *MAT2A* is expressed by all extrahepatic tissues with predominance in the fetal liver, encoding for a catalytic subunit (α2) organized into isoenzyme MATII that exists in polymeric forms different from tissue to tissue. A third gene, *MAT2B*, which encodes for a β-regulatory subunit existing in two distinct splicing variants: V1 and V2 that differ in the amino acid sequence of the NH2-terminal portion. The expression of the *MAT2B* gene was observed only in mammals, specifically in all the extra-hepatic tissues and at low levels in the adult liver (15-16).

Both splicing isoforms of the regulatory subunit β, associated with MATII, regulate its catalytic activity lowering the Km value for methionine and the Ki for AdoMet, thus making

the isoenzyme MATII the most efficient but also the most susceptible to AdoMet-inhibition feedback (15-16).

The three isoforms of AdoMet synthetase, even if they catalyze the same reaction, differ in kinetic and physico-chemical characteristics, regulatory properties and susceptibility to inhibitors. The Km for the substrate methionine is lowest for MATII (4-10 μM), followed by MATI (23 μM -1 mM) and is highest for MATIII (215 μM - 1 mM). The product of MAT activity, AdoMet, is a feedback inhibitor of certain MAT isoenzymes at specific concentrations; MATII has high sensitivity to AdoMet inhibition, with a 50% inhibitory concentration (IC50) of 60 μM, the normal physiological level of the sulfonium compound, whereas it minimally inhibited MATI (IC50=400 μM). On the contrary, MATIII is stimulated at high AdoMet levels (as 500μM) (17).

Given the difference in kinetics and regulatory properties, the type of MAT isoenzyme expressed can influence cellular AdoMet levels. Consistent with this, the intracellular concentration of AdoMet is higher in the cells expressed *MAT1A* gene rather than those expressing *MAT2A*.The presence of several CpG islands on the promoter of the *MAT1A* and *MAT2A* genes has highlighted the existence of an epigenetic mechanism which regulates the expression of such genes. In fact, it has been demonstrated that the switch in the expression of the MAT genes is associated with the hypermethylation of the *MAT1A* promoter gene and simultaneously with hypomethylation of *MAT2A* promoter gene (16-19). Due to kinetic differences and regulatory properties discussed above, a switch from *MAT1A* to *MAT2A* expression results in lower steady state AdoMet level, predisposing the liver to develop steatohepatitis, cirrhosis, and ultimately hepatocellular carcinoma (HCC)(16-19). It has been shown that the enzymatic activity reduction or epigenetic regulation of *MAT1A* gene contributes to pathogenesis and tumor progression in patients affected by liver cirrhosis, causing hyper-methioninemia and delay of plasma clearance of methionine.

Recently, the presence of MAT in the nucleus has been demonstrated (20) and it has been hypothesized that this MAT activity might be involved in providing a source of nuclear AdoMet necessary for DNA or histone methylation.

Although most studies reporting MAT gene involvement in liver cancer, there are increasing reports that *MAT2A* and *MAT2B* are also dysregulated in other cancers, namely: colon, gastric, breast, pancreatic, and prostate cancer (21).

It is known that the sulfonium compound plays a primary role in cellular metabolism, since it represents the link to three key metabolic pathways: transmethylation,

transsulfuration, and polyamine synthesis. In addition, AdoMet is involved as donor of the lateral chain in the biosynthesis of diphthamide, ethylene and in several post-translational modification reactions during tRNA biosynthesis. Furthermore, AdoMet is able to donate the NH2-group of the lateral chain during the biotin synthesis as well as the whole adenosyl portion. The sulfonium compound can also function as allosteric modulator in several enzymatic reactions (1-2) and it is able to mediate novel radical chemical reactions in anaerobic organisms, in which biochemical transformations are regulated by AdoMet radical enzymes (22). In addition, AdoMet in the liver is an important regulator of the glutathione levels, the well-known endogenous antioxidant involved in the prevention of liver diseases (23).

The first known and well studied metabolic reaction since the elucidation of the AdoMet's structure is the transmethylation pathway where the sulfonium compound donates its methyl group to a large variety of acceptor molecules in reactions catalyzed by methyltransferase. In mammals AdoMet is the main methyl donor, while N5-methyltetra-hydrofolate, methyl cobalamin and betaine are implicated as methyl donors only in few reactions, as they are less efficient in transferring the methyl group. The several chemical acceptors of the methyl group from AdoMet are surprisingly high because the transmethylation reactions are involved in the biosynthesis of a wide range of metabolites with consequent formation of N-CH3 bonds (for example coline); S-CH3 bonds (methionine); O-CH3 bonds (phenols etc); and C-CH3 bonds (as several antibiotics) (1-4, 24).

Moreover, experimental evidences in the last years have suggested the involvement of AdoMet-dependent transmethylation reactions in multiple biological processes, such as differentiation, secretion, bacterial chemotaxis, and gene expression (24-26). In addition, AdoMet-dependent transmethylation reactions exerts a key role in several cellular processes, such as DNA/RNA modification and regulation, protein post-translation modification, phospholipids methylation in the membranes and detoxification processes through small molecule methylation (23-25).

About DNA regulation AdoMet mediated, the transmethylation reaction is correlated to resistance to viral infections (27) as well as to a general regulation of gene expression (28). From a quantitative point of view, methylation is the major function of AdoMet since about 95% of the formed sulfonium compound is consumed in such reactions. Approximately 80% of the formed AdoMet is used in the liver for methylation of guanidine acetic acid in the

synthesis of creatine. The variety of compounds and bonds created by enzymatic transmethylations indicates how these reactions perform multiple physiological roles.

S-adenosylhomocysteine (AdoHcy or SAH), is generated as by-product of all transmethylation reactions, and is hydrolized to form homocysteine (Hcy) and adenosine by a reversible enzyme known as AdoHcy hydrolase, whose thermodynamics favours biosynthesis rather than hydrolysis (29). *In vivo* this reaction proceeds as hydrolysis because the products, Hcy and adenosine, are promptly removed. AdoHcy, is a potent competitive inhibitor of methyltransferases and therefore constitutes an important regulatory factor (30). The Ki values of AdoHcy for methyltransferases are similar to ones measured *in vivo*, suggesting that the concentrations may be one of the factors that regulate methylations *in vivo*.

There are two fates for homocysteine: to be remethylated to regenerate methionine, or enter in the transsulfuration pathway to be converted to cysteine. Methionine can be regenerated from Hcy via methionine synthase, an enzyme that requires vitamin B12 as a cofactor and uses 5-methyltetrahydrofolate (5-MTHF) as methyl donor, or using betaine-homocysteine methyltransferase, an enzyme that uses betaine (trimethylglycine), a metabolite of choline. Tetrahydrofolate is generated as a by-product of methionine synthase and is converted to 5-10-MTHF to complete the folate cycle (1-4, 29).

The transsulfuration pathway links AdoMet to cysteine biosynthesis. Here Hcy is converted to cysteine (the limiting reagent in glutathione (GSH) synthesis) through a two-step enzymatic process catalyzed by cystathionine β-synthase and cystathionase, both requiring vitamin B6 (31).

In the liver, AdoMet inhibits methyl tetrahydrofolate reductase (therefore reduces 5-MTHF, the substrate for methionine synthase) and activates cystathionine β-synthase. Thus, depending on AdoMet level, when it is depleted Hcy is channeled to re-methylation to regenerate AdoMet, whereas when the level is high, Hcy is channeled to the transsulfuration pathway. In Figure 3, the metabolic relationships among transmethylation, transsulfuration pathways and the folate cycle are summarized .

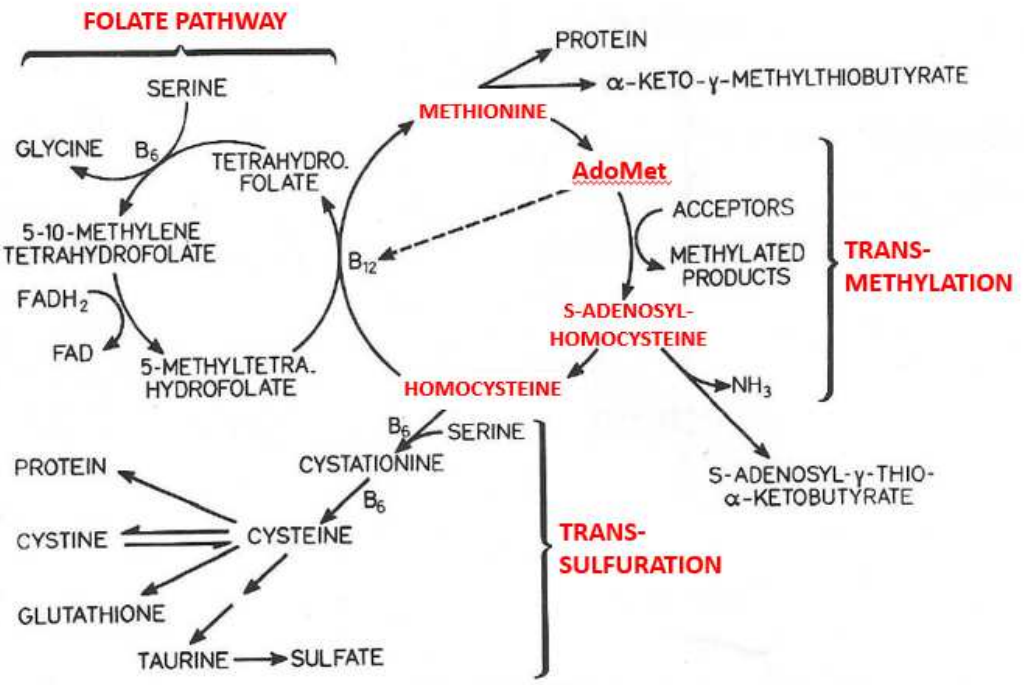

Fig. 3- Metabolic relationships among transmethylation, transsulfuration pathways and the folate cycle.

In addition to its well-recognized role as a methyl donor in transmethylation reactions, AdoMet is a key player in the synthesis of polyamines (Fig.4). Polyamines are low molecular weight organic polycations that are ubiquitous in all living cells. Due to their positive charges, polyamines can bind to macromolecules such as RNA, DNA, and proteins, exerting an essential role in immune response, cell growth, proliferation and apoptosis (1-3, 32).

To enter this pathway, AdoMet needs first to be decarboxylated to S-adenosyl-(5')-3-methylthiopropylamine (dcAdoMet) by AdoMet decarboxylase, which is the rate-limiting step for polyamine synthesis. The predominant polyamines in mammalian cells are spermidine and spermine. As consequence of such biosynthetic reaction, two moles of 5'-deoxy-5'-methylthioadenosine (MTA) are released per mole of spermine and one mole of MTA per mole of spermidine. Therefore, in *Eukarya* polyamine biosynthesis represents the major pathway of MTA formation.

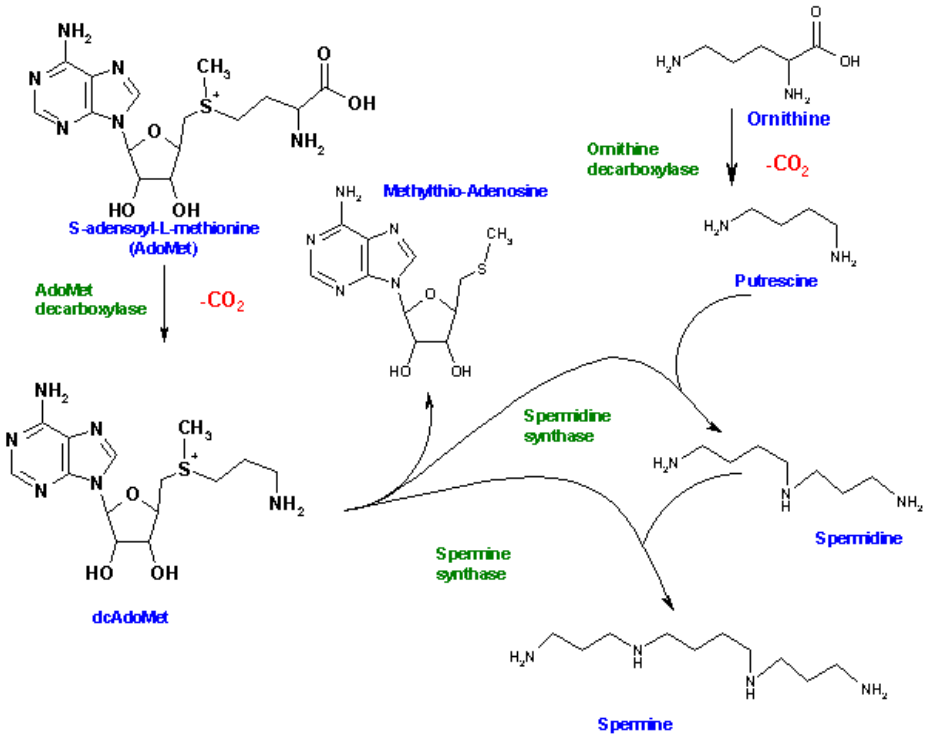

Fig. 4-Polyamine biosynthetic pathway.

A deep study of the literature concerning the physiological and pathophysiological roles of AdoMet, have highlighted the therapeutic potential of AdoMet in the treatment of osteoarthritis, depression, and neurodegenerative disease (33-39) (Fig. 5), suggesting that it acts as anti-inflammatory, anti-depressant and analgesic agent. Clinical trials have demonstrated that AdoMet has the same efficacy in reducing joint pain and inflammation as no steroidal anti-inflammatory drugs (34). There is evidence that AdoMet epigenetically modulates the expression of genes coding for inflammatory mediators, for example, tumor necrosis factor-alpha, interleukin 10, chemokine (C-C motif) ligand 2 (CCL2) and C-C chemokine receptor type 2 (CCR2) (35). AdoMet as antiinflammatory action might be important in the treatment of depression, as inflammation might play a role in its initiation and progression. Furthermore, it has been shown that the sulfonium compound increases brain serotonin levels, acting as an anti-depressant drug, but with fewer side effects (36). Alteration in trans-methylation mechanisms have been described to occur in neurodegenerative diseases, such as Parkinson, Alzheimer as well as in more complex psychiatric syndromes, such as schizophrenia and dementia (40-45). Taken together, the murine (42) and early human studies (43)demonstrate that AdoMet can positively affect hallmarks of Alzheimer disease (presenilin1 expression, β and γ secretase activity, amyloid β generation, phosphotau

accumulation and acetylcholine synthesis), as well as its clinical manifestations (depression, cognition, and aggression). A few studies also suggest a role for the sulfonium compound in cartilage repair, although the underlying mechanisms are not clear (34).

Fig. 5- Therapeutic potential of S-adenosylmethionine.

AdoMet is used for intra-hepatic cholestasis treatment as well as to cure chronic liver diseases due to alcohol abuse (37). Clinical studies have shown that after AdoMet administration to patients with intra-hepatic cholestasis, the liver functions were significantly improved. Serum total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase and gamma-glutamyl transpeptidase levels were significantly reduced within the third week of treatment and came back to normal levels after four weeks, demonstrating the cytoprotective action of the sulfonium compound in acute and chronic liver damage (46). A clinical study in 123 subjects, men and women, with liver insufficiency due to alcohol abuse, demonstrated that treatment with the sulfonium compound for 2 years increased life expectancy and delayed the need of liver transplant (47). Furthermore, it has been shown that patients with cirrhosis had decreased *MAT1A* expression, with consequent accumulation of methionine and significantly reduced AdoMet levels (16,18).

The physiological roles of the sulfonium compound in liver and its therapeutic use in liver disease are reviewed in-depth in the literature (15, 16, 37, 38, 46, 47).

To confirm such positive clinical outcomes and validate new therapies, more studies are needed with a larger population and with longer treatments. Because of its involvement in many physiological and pathological roles, AdoMet can be considered as a structural model to design potential new pharmacological inhibitors. Until today, several pharmacological studies are involved in the development of alternatives and stable AdoMet formulations due to its potential versatility in the treatment of numerous diseases.

In recent years, several new metabolic functions have been assigned to this important and widely occurring sulfonium compound that is now considered a key regulator in diverse cellular processes including proliferation and apoptosis (48-52).

S-Adenosylmethionine and cancer

In addition to multiple functions performed by AdoMet in cellular metabolism, recently many *in vitro* and *in vivo* studies have shown the involvement of the sulfonium compound in various cellular processes, including proliferation, differentiation, cell cycle regulation and apoptosis (3, 17-18, 48-52).

Most of the scientific papers in the literature report data on liver, hepatocarcinogenesis and HCC. AdoMet, the main methyl group donor in the cells, since its discovery, has emerged as a key molecule that plays a central role in numerous hepatic processes. In fact, it was well-known that the impairment in AdoMet biosynthesis may contribute to the pathogenesis of liver disease and predisposition to malignant degeneration. Furthermore, growing evidence has elucidated several molecular mechanisms by which AdoMet exerts its inhibition on hepatocytes proliferation (49-51).

One of the molecular mechanisms by which AdoMet reduces uncontrolled liver cells proliferation seems to be the inhibition of mitogenic signal induced by the hepatocyte growth factor (HGF) (51-53). HGF phosphorylates and subsequently activates the liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK), whose activation promotes the translocation from the nucleus to the cytosol of HuR (human antigen R); in the nucleus this protein stabilizes several cell-cycle genes such as cyclin A2 and D1, allowing the hepatocytes to proliferate (51). AdoMet, through the methylation of protein phosphatase 2A (PP2A) and its association to AMPK, is able to prevent this process by inhibiting the phosphorylation of AMPK (52-53). Consequently, HuR is not transported to the cytoplasm and the stabilization of target genes does not occur, so the half-life of the mRNAs encoding the cyclin A2, D1 and D2 (52-53) and p53 and ubiquitin carboxyl-terminal hydrolase 7 (USP7) (51) are increased.

Tomasi *et al.*, have proposed a different mechanism by which AdoMet cytoplasmic levels affect the hepatic cell growth. Extracellular signal regulated kinase (ERK) activation is one of the several growth signals associated with highly malignant HCC phenotypes and it is tightly regulated in normal liver cells. One way through which ERK activity is kept under control is by the action of the dual-specificity MAPK phosphatase (DUSP1). DUSP1 deregulation and the ERK1/2 overexpression induce a rapid HCC progression in humans (54). AdoMet stabilizes DUSP1 that can dephosphorylate both serine/threonine and tyrosine residues of ERK1/2, controlling the MAPK signaling activity (54). It has been shown that in the liver of MAT1A-KO mice, mRNA and protein levels DUSP1 are significantly reduced, whereas AdoMet treatment increases the stability of the DUSP1 mRNA and its protein levels. AdoMet normalized DUSP1 mRNA level likely by enhancing p53 binding to its consensus element in the DUSP1 promoter or through its degradation by proteasome. These results are in agreement with the observation that the AdoMet antiproliferative effect is mediated by the inhibition of ERK1/2 (54).

Several abnormal pathways are responsible for HCC development. During the hepatocarcinogenesis, global DNA hypomethylation induces genomic instability (GI), which is the result of chromosomal rearrangements, duplications, genomic mutations, deletion, replication errors and global DNA hypomethylation (55). Therefore, multiple DNA repair mechanisms exist and ensure that these alterations are constantly and efficiently repaired in normal cells, defending against GI; one of the most critical is the apurinic/apyrimidinic endonuclease 1 (APEX1), which is a multifunctional protein possessing both DNA repair and redox regulatory activities.

Different experimental data could explain the chemopreventive action of AdoMet and why its chronic deficiency predisposes to to hepatocellular carcinoma (55). It is well known that in MAT1A-KO mice, which show chronic hepatic AdoMet deficiency, AdoMet depletion destabilizes APEX1 protein and increased GI, possibly facilitating malignant degeneration (55).*In vitro* experiments performed on human and murine hepatocytes demonstrated that AdoMet stabilizes APEX1 protein, probably preventing its proteosomal degradation.

The development of HCCis also associated with a sharp increase in polyamine synthesis, due to a progressive upregulation of the *ODC* gene (encoding the ornithine decarboxylase), that can lead to fast proliferation of preneoplastic and neoplastic liver cells (56-57). It has been shown that the sulfonium compound may interfere with polyamine synthesis, and consequently cell proliferation, by inhibiting ODC activity(56-57).

AdoMet also regulates hepatocytes death response. Yang *et al*., demonstrated that AdoMet induces apoptosis in HepG2 and HuH-7 hepatocellular cell lines by activating the mitochondrial death pathway (58). The pro-apoptotic effect of AdoMet is accompanied by the release of cytochrome c involving in the regulation of the pro-apoptotic Bcl-xS isoform. Bcl-x is alternatively spliced to produce two distinct mRNAs encoding for the proteins Bcl-xL and Bcl-xS, that have antagonist function, anti-apoptotic and pro-apoptotic, respectively. The sulfonium compound selectively induces Bcl-xS synthesis by activating SR protein–mediated alternative splicing. This occurs through the activation of protein phosphatase 1 (PP1), which leads to dephosphorylation of SR proteins, necessary in the Bcl-x splicing reaction. This effect has not been verified in primary cultures of hepatocytes (58).

HCC development is often associated with epigenetic changes that affect the *MAT* genes expression at both transcriptional and post-transcriptional levels (59-61). Recent studies demonstrated that in the adult liver, under pathological conditions, a switch *MAT1A-MAT2A* could occur (reduction *MAT1A* gene expression and concomitant *MAT2A* gene over-expression) with consequent reduction of the physiological AdoMet levels.

In HCC cells, *MAT1A/MAT2A* switch is associated with global DNA hypomethylation, reduction in DNA repair, genomic instability, and signaling upregulation including c-MYC overexpression, upregulation of RAS/ERK, IKK/NF-kB, PI3K/AKT and finally rise in polyamine synthesis. Furthermore, decrease in *MAT1A* expression and AdoMet levels are associated with increase in HCC cell proliferation, cell survival, and microvascularization. All of these changes are reversed by AdoMet treatment *in vivo* or forced *MAT1A* overexpression or *MAT2A* inhibition in cultured HCC cells. Therefore, the *MAT1A/MAT2A* genes expression ratiois pathogenetically important, infact it could act as a HCC prognostic biomarker, while the regulation of the genes encoding the isoenzymes MAT can represent a potential therapeutic target (59-60).

Moreover, accumulating evidence indicates that a class of mRNA-binding proteins (RBPs) plays a pivotal role in post-transcriptional deregulation of gene expression inHCC. Among RBPs, AU-rich element RNA-binding protein 1 (AUF1) reduces the stability of *MAT1A* mRNA, while HuR selectively binds to AU rich elements, promoting the stabilization of *MAT2A*mRNA (19, 61).

Li and collaborators have investigated the mechanism by which AdoMet exerts its antiproliferative effect in colon and liver cancer. The sulfonium compound is able to block the Wnt/βcatenin signaling pathway through two different mechanisms. In colon cancer cells

(SW480) and liver cells (HCT116), with constitutively active β-catenin signaling, the treatment with AdoMet inhibits β-catenin activity excluding it from the nuclear compartment (62). In colon cancer (RKO) and liver (Huh-7) cells, expressing wild-type Wnt/β-catenin, the sulfonium compound leads to β-catenin degradation in the proteasome by a mechanism glycogen synthase kinase-3β (GSK-3β) mediated (62).

The pleiotropic effects of AdoMet treatment during hepatocarcinogenesis are summarized in Figure 6: AdoMet stabilizes APEX1 reducing GI; AdoMet inhibits the LKB1/AMPK axis increasing HuR cytoplasmic concentration that stabilizes *p53*, *USP7*and *cyclins* mRNAs; AdoMet inhibits RAS/ERK pathway by activating PP2A and DUSP1 and interferes with the cell cycle inhibiting polyamines biosynthesis.

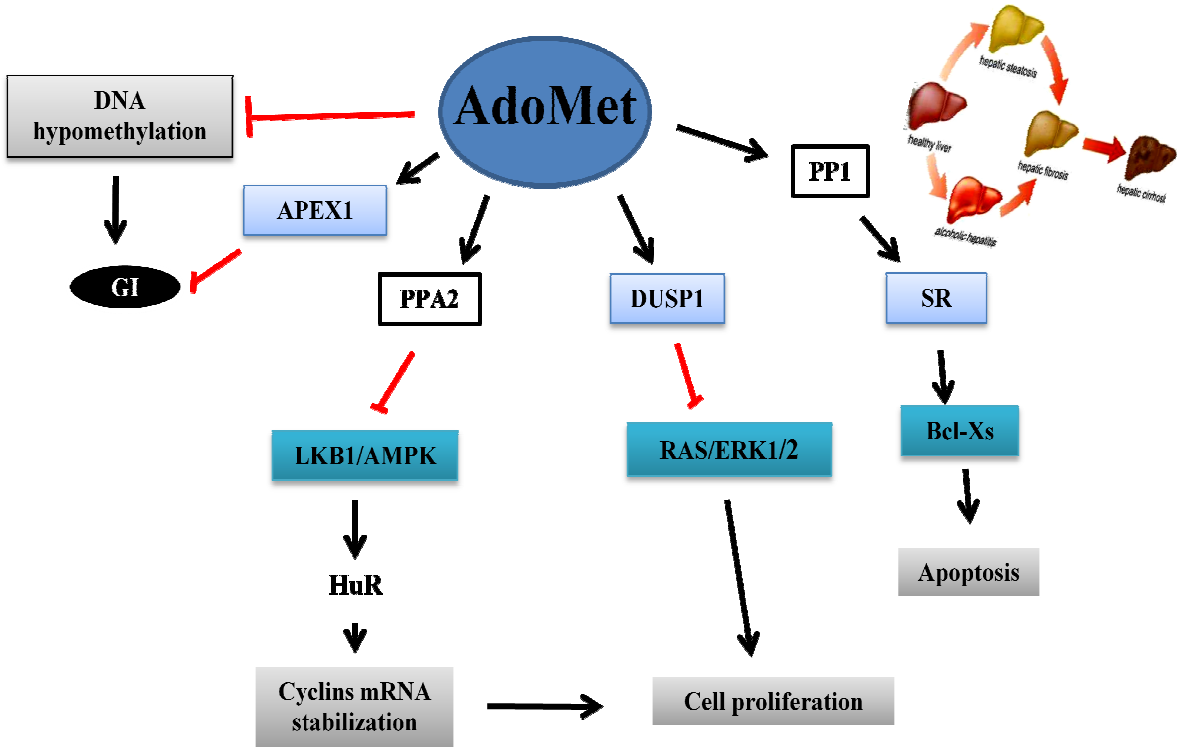

Fig. 6 - Pleiotropic effects of AdoMet treatment during hepatocarcinogenesis.

Apart from AdoMet action on liver cancer cells, recent studies have highlighted the ability of AdoMet to counteract the progression of many other human tumors, although with different mechanisms (63-71).

In colon cancer, AdoMet was able to induce apoptotic process and reduce the mechanism of inflammation (63). Li *et al*., report that AdoMet treatment of RKO and HT-29 colon carcinoma cells reduces the expression of several anti-apoptotic genes, such as the gene encoding FLICE-like inhibitory protein (cFLIP) (63). cFLIP is a protein homologous to caspase 8 but lacks of catalytic domain. Therefore, cFLIP can dimerize with caspase 8, forming an inhibitory complex that inhibits apoptosis. Moreover, the AdoMet treatment leads to the activation of caspase 8, leading a proteolytic cut of the Bid protein, which in turn activates Bid protein through a proteolytic cut, leading to cytochrome c release from the mitochondria and to apoptosome formation, a complex involved in the activation of the apoptotic process (63).

It has been shown that AdoMet and its metabolite MTA reduce the inflammation-induced colon tumor multiplicity, size and tumor load by inhibiting fundamental pathways involved in colon carcinogenesis (64). In Balb/c mice with drug-induced colon cancer, AdoMet and MTA treatment reduced tumor load of about 40%. These treatments induced apoptosis and inhibited many signaling pathways important in colon carcinogenesis, including β-catenin, IL-6 signaling, pro-inflammatory cytokine, IL-1β and several pro-growth and proliferation pathways (64).

The phenotype of a tumor cell is the result of a series of genetic and epigenetic events (activation of oncogenes, inactivation of tumor suppressor genes, translocations, hypermethylation, hypomethylation, etc.). Changes in the methylation pattern in the region of gene promoters are one of the ways of regulating gene expression at the transcriptional level.

Zhao *et al*. demonstrated that AdoMet treatment inhibits, in a dose- and time-dependent manner, the growth of SGC- 7901 and MKN-45 gastric cells through a down-regulation in the expression of urokinase-type plasminogen activator (uPA) and *c-MYC* genes, by partly or completely methylation of these genes. *In vivo* AdoMet treatment reduces the volume of gastric xenografts tumors in male Balb/c nu/nu mice (65).

It has also been shown, that in MGC-803 human gastric cancer cells and in HT-29 colon carcinoma cells, *c-MYC* and *H-RAS* oncogenes are in hypomethylated state so their expression levels are particularly high. AdoMet is able to effectively induce the DNA methylation in the promoter of such oncogenes, inhibiting protein expression and consequently reducing tumorigenesis. It is interesting to underline thatin normal cells, oncogenic *c-MYC* and *H-RAS* are supposed to be hypermethylated, therefore AdoMet treatment should not have any effect on their methylation pattern (66).

Moreover, other findings suggest that AdoMet is effective in the primary stage of osteosarcoma (67). In human LM-7 and MG-63 osteosarcoma cells, AdoMet treatment leads to a dose-dependent decrease of cell proliferation and invasiveness of tumor cells. The mechanism is mediated by the lower expression of Sox2 and by an up-regulation of translational-controlled tumor protein, both proteins linked to the proliferative ability of normal bone tissue (67).

In addition, our research group has recently showed that AdoMet strongly inhibits proliferation of U2OS osteosarcoma cells by slowing-down cell cycle progression, causing a reduction in protein expression levels of cyclin D and E, an increase of p53 and p21 cell-cycle inhibitor, as well as by triggering apoptosis (68). The AdoMet-induced antiproliferative effects were dynamically accompanied by profound changes in protein and phosphorylation levels of ERK1/2 and STAT3 (68).

Furthermore, our research group has deeply investigated AdoMet antiproliferative effect on different hormone-dependent and -independent breast cancer cell lines and has proposed the possible cell death mechanism responsible for the synergistic effect of the combination of the sulfonium compound with doxorubicin (Doxo), one of the most one of milestones in the treatment of breast cancer (69-71). The mechanisms of cell death induced by the synergistic combination AdoMet/Doxo has been evaluated in hormone-dependent CG5 cells, with special interest upon programmed cell death. AdoMet/Doxo combination induced a significant activation of the extrinsic apoptotic due to increase of Fas death receptor and its ligand expression (FasL). These results highlight the importance of the synergistic effect of AdoMet with Doxo in the regulation of hormone-dependent breast cancer cell proliferation and emphasize the anti-tumor activity of these molecules (69).

In Figure 7 is summarized the different mechanism AdoMet mediated, by which the sulfonium compound exert its antiproliferative, pro-apoptotic and antimetastatic effects.

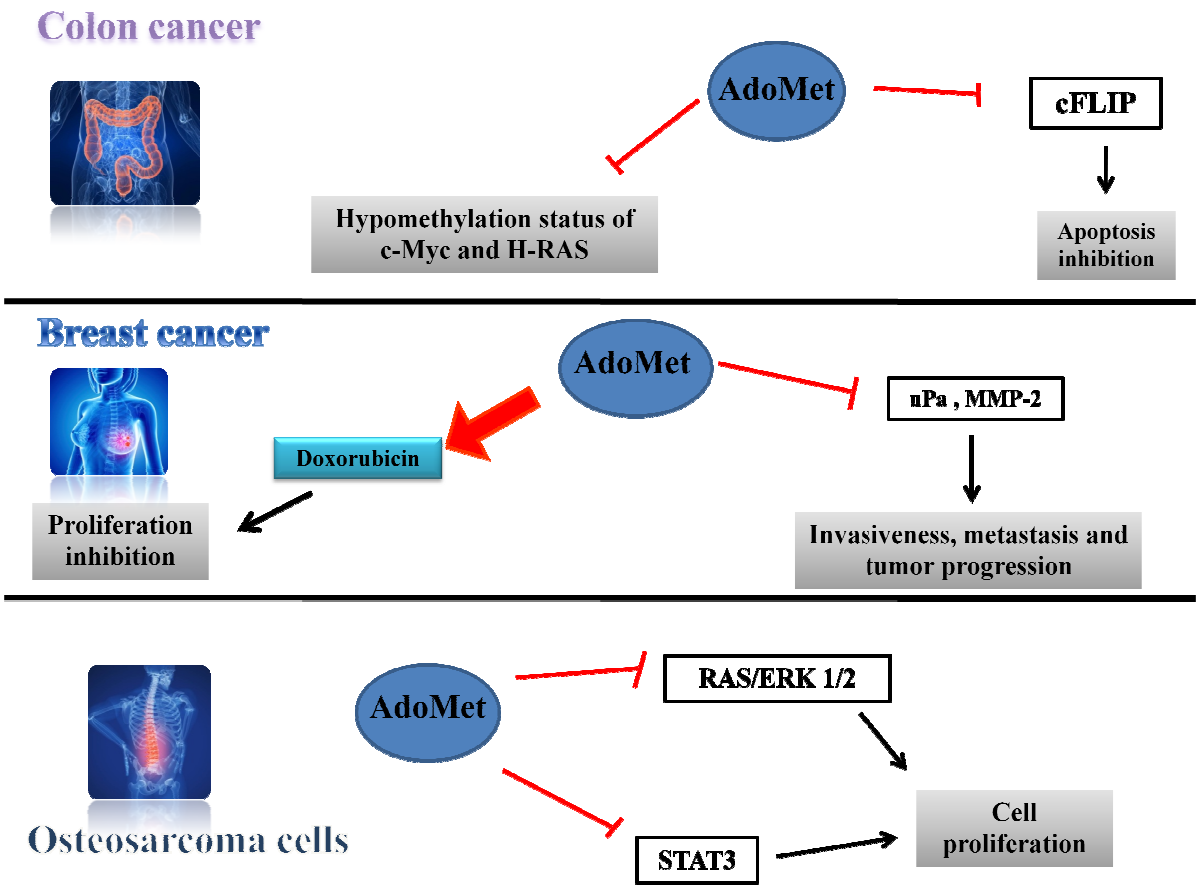

Fig. 7 - AdoMet's ability to counteract the progression of many human tumors by different mechanisms.

In MCF-7 cells, AdoMet fulfills a strong inhibitory effect on cell proliferation by inducing both autophagy and apoptosis. AdoMet consistently enhanced the levels of the autophagy markers beclin-1 and LC3B-II, and caused a significant increase of pro-apoptotic Bax/Bcl-2 ratio paralleled by Poly (ADP ribose) polymerase (PARP) and caspase 9, and 6 cleavage. Notably, AdoMet, already at low doses, raised the percentage of cells in G_2/M phase of cell cycle by down-regulating the expression of cell cycle-regulatory proteins cyclin B and cyclin E with a remarkable increase of p53, p27, and p21. We also demonstrated that the combination of AdoMet and the autophagy inhibitor chloroquine (CLC) potentiates apoptosis occurrence (Fig. 8) (70).These effects were paralleled by a strong inhibition of the activity of AKT and of the downstream effector mTOR and by an increased cleavage of caspase-6 and PARP. These data suggest, for the first time, that autophagy can act as an

escape mechanism from the apoptotic activity of AdoMet, and that AdoMet could be used in combination with CLC or its analogs in the treatment of breast cancer (70).

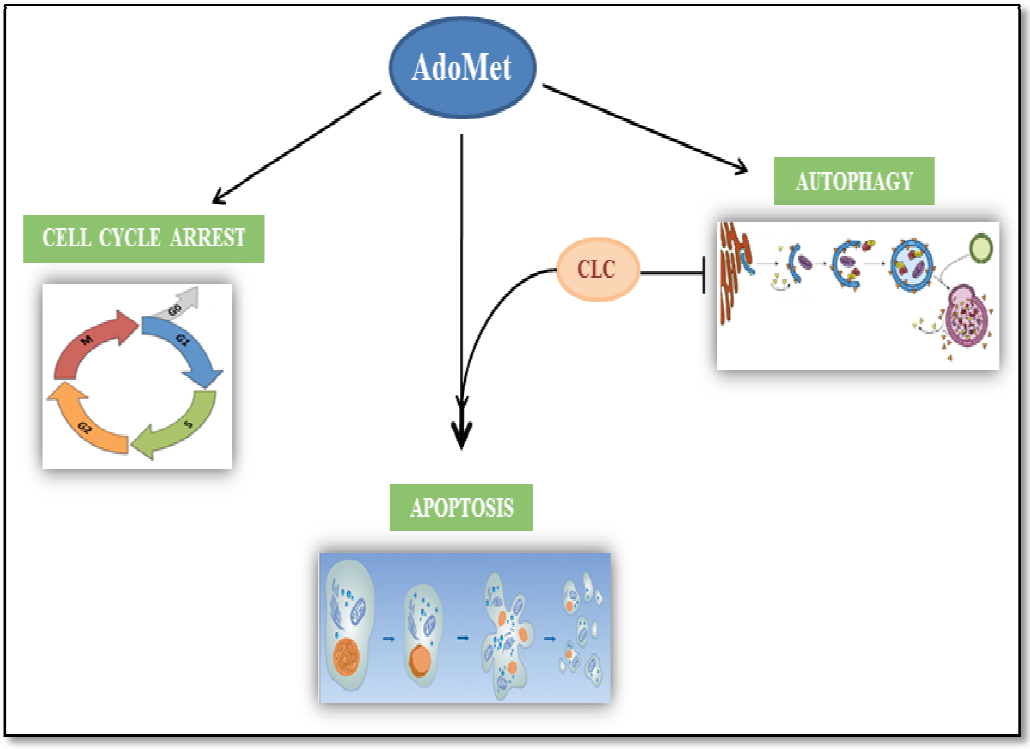

Fig. 8 - Antitumor effects of AdoMet on MCF7 cells. *Source: The Anticancer Effects of S-Adenosylmethionine on Breast Cancer Cells. JSM Chemistry, 2333-6633., 2017. Reference n.71.*

Emerging evidences document the involvement of AdoMet in the regulation of genes responsible of cell invasion and metastasis (72-76) and many research groups studied in depth the epigenetic regulation induced by AdoMet on methylation status of genes involved in the invasion and metastases processes.

Chik and colleagues demonstrated that AdoMet synergizes with the DNA methylation inhibitor 5-aza-2-deoxycytidine to suppress uPA expression, thereby blocking MDA-MB-231 cell invasiveness (72).

It has been shown that the treatment of in highly invasive MDA-MB-231 breast cancer cells as well as PC-3 prostate cancer cells with AdoMet significantly inhibits uPA and MMP2 expression resulting in a strong inhibition of tumor cell invasion *in vitro* and tumor growth and metastasis *in vivo* (73-74).

More recently, it was been reported that AdoMet is able to enhance the anti-metastatic effect of gemcitabine in pancreatic cancer through the inhibition JAK2/STAT3 pathway (75) and in association with selenium compounds in human cervical cancer HeLa cells, inhibits cell proliferation, migration and adhesion, by affecting ERK and AKT signaling pathways (76).

It is interesting to note that AdoMet is available as a dietary supplement in the United States since 1999 and pharmaceutical preparations of this compound are available as intravenous, intramuscular, and oral forms. Reviews of clinical studies to date indicate that, at pharmacological doses, AdoMet has a low incidence of side effects with an excellent record of tolerability. Moreover, no toxic or antiproliferative effects have been reported in normal, non tumorigenic cells. Thus, it is conceivable that the concentrations of AdoMet that would inhibit cancer cell proliferation, used in our as well as in other studies, could be useful for therapeutic purpose.

Laryngeal Squamous Cell Carcinoma

Laryngeal Squamous Cell Carcinoma (LSCC) represents approximately one-third of all Head and Neck Squamous Cell Carcinoma (HNSCC) and due to its highly aggressive features, is considered a major cause of cancer-related deaths, with more than 150,000 new cases diagnosed annually.

The diagnosis of LSCC is made according to the World Health Organization (WHO) classification from a surgical biopsy sample (77-80).

The larynx extends from the tip of the epiglottis to the inferior border of the cricoid cartilage. The vast majorities of malignant neoplasms of the larynx arise from the surface epithelium and are therefore classified as keratinizing or non keratinizing squamous cell carcinomas. Laryngeal cancers are divided into supraglottic, glottic, and subglottic subsites, with pathophysiology and treatment differing according to the subsite.

Several risk factors have been implicated in the pathogenesis of laryngeal cancer.The most significant of these are tobacco and alcohol consumption (77). A dose-dependent effect of cigarette smoking has been observed, and the risk for cancer remains elevated even several years after smoking cessation. Exposure to several other environmental factors is thought to potentially increase the risk of LSCC, such as asbestos, polycyclic aromatic hydrocarbons, and textile dust. Dietary factors have also been noted, with red meat increasing the risk of laryngeal cancer, while a diet varied in fruit and vegetables potentially has a protective effect. Other risk factors, including human papilloma virus (HPV) and laryngopharyngeal reflux, remain under investigation and their link to laryngeal cancer is controversial (78-79).

Given the unique physiological function of the larynx, there are several common presenting symptoms such as hoarseness, dysphonia, dyspnea, and swallowing dysfunction.

Patients who suffer with laryngeal cancer are commonly in older age group, with a long-term survival rate of less than 50%. Males are approximately three times more likely to develop LSCC than females.

Laryngeal carcinoma infiltrates locally in the mucosa and beneath the mucosa and can metastasize via the lymphatic system and the bloodstream. If it spreads, the cancer has a worse prognosis and can be fatal. Prognosis has improved little in the past 30 years (78). About half of affected individuals survive more than five years after diagnosis.

Treatment is predominantly based on the stage of the disease. Factors such as patient fitness, baseline swallow, airway functional status, and others are considered before determining the treatment plan (81-82).

Over the past three decades, laryngeal cancer treatment has changed drastically, moving from the classic laryngectomy approach to the combination with chemotherapy and radiotherapy. The surgery and radiation is usually used in combination, because the radiation can be destroyed small areas of cancer that cannot be removed surgically. In general, the early-stage patients retain vocal and swallowing functions after treatment. However, advanced patients often suffer from permanent tracheostomy and loss of natural voice. Sometimes before radiotherapy is given chemotherapy to shrink the tumor and help to make the radiotherapy more effective. Chemotherapy drugs commonly used to treat HNSCC are agents such as cisplatin, carboplatin, docetaxel and 5-flurouracil (81). Targeted molecular therapies were developed since the discovery of role of epidermal growth factor receptor (EGFR) signaling in HNSCC development, progression and prognosis. These targeted therapies include monoclonal antibodies (such as cetuximab, panitumumab etc.) and tyrosine kinase inhibitors(such as erlotinib, gefitinib, etc.). One of the main risks of targeted therapy is that the cancer cells can become immune to the treatments.

Cisplatin-based chemotherapy is the mainstay therapy for LSCC. However, intrinsic or acquired drug resistance during the course of the treatment is the major limitation of its usage (82). The proposed mechanisms of resistance include changes in cellular uptake and efflux of cisplatin, increased biotransformation and detoxification in the liver, and increase in DNA repair and anti-apoptotic mechanisms (82). Despite the progress made in treatment regimes, the 5-year mortality rate has not changed, remaining around 50%. This is due to high toxicity induced by the drugs used for the treatment of LSCC, and to occurrence of drug resistance mechanisms, which limit their use.

In this regard, there is a strong interest to identify and develop new LSCC therapies that take advantage of the combination of natural compounds and chemotherapeutic molecules currently used for LSCC (83-86).

Baharuddin *et al*. studied the effect of curcumin in non-small cell lung cancer (NSCLC) cell lines (A549 and H2170), and they found that curcumin sensitized those cell population to cisplatin treatment by downregulating cyclin D1 and activating p21/Apaf/caspase-9 pathway. Therefore, the treatment with a combination of curcumin and cisplatin induced cell cycle arrest resulting in the initiation of the intrinsic apoptotic pathway (83).

Moreover, the combination of curcumin and cisplatin enhanced growth suppression in HNSCC, through two different molecular pathways, involving NFkB, a regulator of genes

that control cell proliferation and cell survival. Curcumin affects NFκB transactivation by inhibiting IKK kinase activity both in the cytoplasm and nucleus, possibly through an interaction with IKKβ. It was hypothesized that nuclear IKKβ in combination with histone H3 is involved in chromatin remodeling of the NFkB transcription binding sites. Inhibition of IKKβ by curcumin therefore results in reduction in NFkB occupied sites in chromatin leading to a decrease in NFkB mediated transcription. Cisplatin, in contrast, downregulates NFκB through a p53 mediated pathway (84).

Liu *et al.* evaluated the therapeutic effect of the combination Berberine (BBR), a compound extracted from a variety of medicinal herbs, and cDDP. The result showed that the combination of these two compound inhibited cell proliferation and induced apoptosis and necroptosis even more strongly, when compared with either BBR or cDDP treated alone as evidenced by the increased apoptotic and necrotic numbers, higher expression and activation of Caspase 3, Caspase 8, and other necrotic markers (85).

Other findings have also shown that quercetin acts in synergy with ribavirin and cisplatin and can overcome cisplatin resistance in a patient with ovarian cancer and with hepatoma (86).

The importance of synergistic combination of chemotherapeutic agents with natural compounds is represented by the improvement of therapeutic efficacy by reducing side effects and the drug resistance mechanisms.

MicroRNAs

MicroRNAs (miRNAs) are a class of small, endogenous non-coding transcripts of 16 to 29 nucleotide RNAs that function as the universal specificity factors in post-transcriptional gene silencing. They are expressed from longer transcripts encoded in animals, plants, viruses and single-celled eukaryotes (87-90).

MiRNAs regulate target mRNAs for degradation or translation repression, consequently their dysregulation can lead to human diseases. Recent studies have reported differentially regulated miRNAs in diverse cancer types, such as breast cancer (91-92), lung cancer (93), prostate cancer (94), colon cancer (95) and head and neck cancer (96).

In addition, miRNAs are secreted into extracellular fluids. Extracellular miRNAs have been widely reported as potential biomarkers for a variety of diseases and they also serve as signaling molecules to mediate cell-cell communications. Thus, identifying miRNAs, targets and their functional regulatory networks are critical in understanding normal biological processes of miRNAs and their roles in the development of disease (90).

The first small RNA, lin-4, was discovered by Rosalind Lee and Rhonda Feinbaum in 1993, through a genetic screening in nematodes and itregulates larval development through translational repression of LIN-14protein. They found that *lin-4* transcripts in *C.elegans* contain sequences complementary to a repeated sequence element in 3' untranslated region of *lin-14* mRNA. *Lin-4* regulates *lin-14* through RNA-RNA interactions with the 3' UTR. When *lin-4* was expressed the disappearance of the LIN-14 protein was assisted (97).

In 2000, a second miRNA, let-7, was discovered, and since then thousands more miRNAs have been identified (98).

In most recent version, miRBase reports ~ 2600 human miRNAs (version 22: www.mirbase.org 2018) and current estimates suggest that over 30% of human genes are regulated by miRNAs (99), leading to different effects on multiple cellular processes.

MiRNA genes can be classified into four groups according to their genomic location: intronic miRNA encoded in non-coding transcriptional units; exonic miRNA encoded in non-coding transcriptional units; intronic miRNA encoded in protein-coding transcript units, and exonic miRNAs encoded in protein-coding transcripts. Nearly half of the known human miRNAs are found in clusters, which are transcribed as polycistronic primary transcripts (100).

Biochemically, the biogenesis of miRNA starts with the transcription of a miRNA gene by RNA polymerase II (101). The mRNA transcript at this stage is called primary

transcript RNA (pri-miRNA) which forms a stem loop structure of 70–100 nucleotide by the action of double-strand (ds)-RNA-specific ribonuclease (Drosha) and its binding factor DiGeorge syndrome critical region gene 8 (DGCR8) (102). This structure is called precursor miRNA or pre-miRNA which is transported to cytoplasm by Exportin-5-RanGTP dependent mechanism where they are further processed to become mature miRNAs by dsRNA specific ribonuclease (DICER) (103-104). Dicer is a highly conserved and specific enzyme which firstly recognizes the double-stranded portion of the pre-miRNA, with a particular affinity the 5’ phosphate and 3’ overhang at the base of the stem loop and which cut both strands of the duplex, generating an approximately 22~nucleotides miRNA duplex. Following cleavage by Dicer, the miRNA duplex interacts with the Argonaute protein (AGO) and then is incorporated as single-strand RNA into a ribonucleoprotein complex, known as the RNA-induced silencing complex (RISC) (105).

The AGO family proteins are composed by three distinctive domains: the PAZ, MID and PIWI domains. The PAZ and the MID domains bind to the 3’ and the 5’ ends of small RNA, respectively, and in particular PAZ also interacts with the PIWI domain, which has a structure similar to the ribonuclease H (RHase H) and is able to cut the RNA strand (106). Primarily one strand of the miRNA duplex (passenger strand) is degraded, whereas the other strand (guide strand) is preferentially assembled into the complex. Mature miRNA results from this process which is bound to a complex. The whole complex is called miRNA-associated RNA-induced silencing complex (miRISC) and downregulate gene expression by two posttranscriptional mechanisms: mRNA cleavage or translational repression. The degree of miRNA-mRNA complementarity is the major determinant of the regulatory posttranscriptional mechanism.

MiRNAs that bind to mRNA targets with imperfect complementarity at the sites located within the 3′ UTR of the mRNA gene block target gene expression at translational level. MiRNAs that bind to their mRNA targets with perfect (or nearly perfect) complementarity at the sites generally found in the coding sequence or open reading frame (ORF) of the mRNA target induce target mRNA cleavage (106). Figure 9, summarizes all the steps of miRNAs biogenesis (107).

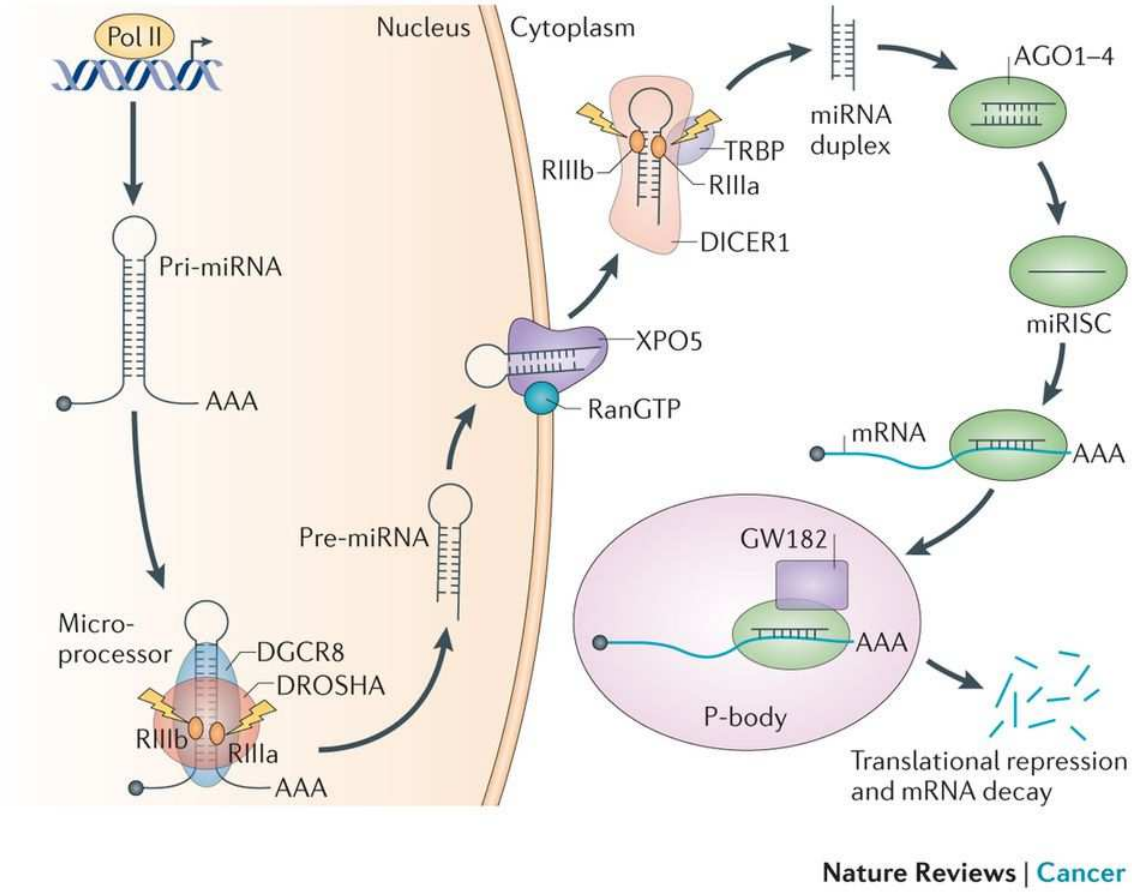

Fig.9 - MiRNA biogenesis *Source: Shuibin Lin and Richard I. Gregory; MicroRNA biogenesis pathways in cancer. (2015) Nature Reviews Cancer, pg321–333. Reference n.107.*

MiRNA binding sites have also been detected in other mRNA regions including the 5′ UTR and coding sequence, as well as within promoter regions (108). The binding of miRNAs to 5′ UTR and coding regions have silencing effects on gene expression (109) while miRNA interaction with promoter region has been reported to induce transcription (110-111). However, more studies are required to fully understand the functional significance of such mode of interaction.

Recent studies have shed light on the dynamic nature of miRNA actions and further revealed the complexity of miRNA-mediated gene regulation. Many factors contribute to the activity of miRNAs, including subcellular location, miRNA/mRNA abundance, cell type/state, and the availability of different miRISC components. miRNAs in the nucleus play a role in regulating transcription and alternative splicing (90).

Although a lot of research has been conducted on the role of miRNAs in disease development and therapy, still the basic mechanism of action of miRNAs is not fully understood.

MiRNAs and tumorigenesis

It has been shown that generally in human neoplasms there is a global reduction of total miRNA levels (112). In fact, about 50% of the genes encoding them are found in chromosomal regions frequently subject to mutation in tumors (called fragile sites) (113). Genes that code for miRNAs can be subject to the same types of mutations that occur in protein-coding genes (point mutations, deletions, amplifications), which can inhibit or promote expression. They can also be epigenetically regulated by promoter methylation .Next to mutations, genetic or epigenetic, the expression of miRNAs can also be indirectly regulated by mechanisms of transcriptional control, in fact the transcription of miRNAs is regulated by proteins, whose expression or function is frequently altered in tumors (as p53, c-MYC). If the expression of a given transcription factor is induced during tumor transformation process, or abnormally inhibited, this results in aberrant activation or inhibition of the transcription of all the genes that this factor controls, among which the miRNA genes. In these cases, the expression of the miRNA can be inserted in a positive feedback mechanism, contributing to keep active the signal path responsible for its induction, or in a negative feedback mechanism, able to oppose the action. Another indirect mechanism to control the expression of miRNAs regards the onset of mutations in the genes coding for the proteins involved in the miRNA biogenesis (including DICER, DGCR8); for example, the loss of function of an allele encoding DICER1 was shown to contribute to a global decrease in cellular miRNA levels and favoured transformation and neoplastic progression (113-115).

Considering that each miRNA can regulate even one hundred or more target genes, it is clear that the aberrant expression of a single miRNA can cause consistent changes in the cell gene expression and therefore in its signal pathways. Because the aberrant expression of miRNAs is often involved in neoplastic processes, these molecules are interesting targets for the development of new anti-tumor therapies.

The interest in the study of therapeutic strategies that go to act on miRNAs is linked to the fact that by intervening therapeutically on a single molecule it is possible to obtain multiple effects on the gene expression of the cell, as each miRNA regulates very many target genes.

There are two approaches in this area: induction of oncosuppressor miRNA expression and inhibition of oncogenic miRNA (oncomiRs) expression. MiRNAs with tumor suppressor function, which are generally under-expressed in tumors, can be used as therapeutic molecules to cause cancer cells to die. On the contrary, to inhibit the expression of oncomiRs it is possible to use molecules able to bind stably the miRNA, subtracting it from its biological function: Since each cell line expresses different endogenous miRNA levels it is important to choose carefully whether in an experiment it is necessary to simulate or attenuate their effect on target gene expression using miRNA "mimic" or "inhibitor", respectively. Mimic miRNAs, since they reduce the expression of target genes, are more used in cells that express a low level of endogenous miRNA and therefore have a high expression of target genes. Instead, cells with high levels of endogenous miRNAs and low expression of target genes are the most widely used to study the effects of inhibitors, which bind to endogenous miRNA to prevent it from repressing gene expression.

The antimiRNA or miRNAs inhibitor are single-stranded, antisense oligonucleotides designed to inhibit miRNA function. The perfect complementarity of the sequence allows the antisense oligonucleotide to bind to the miRNA and interfere with its function. Mimic miRNAs are short double-stranded oligonucleotides in which one strand is identical to the mature miRNA sequence (guiding strand) and the other strand is complementary or partially complementary and complexed with the mature miRNA sequence (passenger filament). The double-stranded structure for recognition and loading of the guiding strand in the RISC is required, after which it can function as endogenous miRNAs to increase the level of miRNA of interest and to block targeted gene expression more efficiently (Fig. 10).

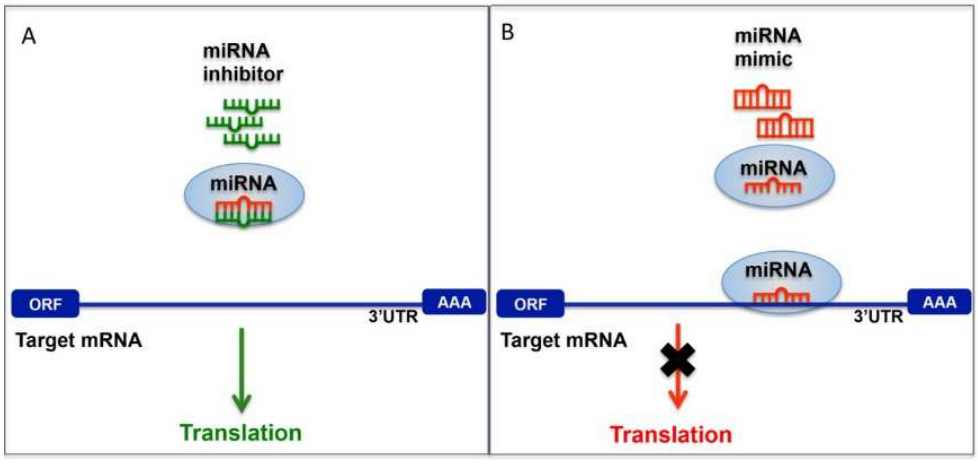

Fig.10 - Schematic representation of miRNA inhibition and mimic assays.

In the light of these considerations, the use of miRNA mimic or inhibitor must be considered a unique opportunity to develop novel therapeutic strategies.

MiRNAs and AdoMet

As already mentioned, AdoMet is the principal methyl donor in all living cells and a key hepatic regulator. The liver is the principal organ for conversion of dietary methionine into AdoMet and where up to 85% of all transmethylation reactions. A lot of miRNA has been correlated to methionine metabolism and liver injury. Recent studies reported the capability of miRNAs to regulate the expression of *MAT1A* gene in HCC, resulting in decreased AdoMet levels and deregulation of signal transduction pathways linked to methionine metabolism and MAT activity (116)

The miR-664, miR-485-3p and miR-495 knockdowns in HepG2 and Hep3B hepatocarcinoma cells induce *MAT1A* expression and reduce cell growth. In mice models, subcutaneous and intraparenchymal injection of Hep3B cells, stably expressing these miRNAs, promotes tumorigenesis and metastasis formation. Treatment of these murine models with siRNA directed against miR-664, miR-485-3p and miR-495 showed a reduction in tumor growth and metastasis formation (116).

It has been also demonstrated that miR-22 and miR-29b critically contribute to the inhibition of *MAT1A* and *MTHFR* gene expression during 2-acetylaminofluorene-induced rat hepatocarcinogenesis. The downregulation of *MAT1A* and *MTHFR* genes is accompanied by reduction of AdoMet and AdoMet/AdoHcy ratio and by the alteration of DNA and histone methylation state, thus causing the promotion of liver carcinogenesis (117).

To date, a direct correlation between the antiproliferative effect of AdoMet and the variation of miRNAs expression has never been shown.

My group of research have demonstrated for the first time that AdoMet was able to modify miRNAs expression profile in MCF-7 breast cancer cell lines. AdoMet treatment significantly modulated three miRNAs, up-regulating miR-34a and miR-34c in both cell lines and down-regulating miR-486-5p expression. Transient transfection experiments showed that the combination of either miR-34a or miR-34c mimic with AdoMet strongly potentiated the pro-apoptotic effect of the sulfonium compound, by activation of a caspases-dependent mechanism and activates p53 acetilation by inhibiting Sirtuin1 and Histone Deacetylase1 expression.

Moreover, miR-486-5p inhibitor enhances the pro-autophagic effect of AdoMet by increasing PTEN expression and by inhibiting AKT signaling (92).

Discovering miRNAs, identifying their targets and further inferring miRNA functions have been a critical strategy for understanding normal biological processes of miRNAs and their roles in the development of disease, like cancer. In fact, miRNAs are emerging as potential diagnostic, prognostic and therapeutic tools for cancer treatment and it is believed that modulating miRNA expression may represent a potential therapeutic strategy for treating cancer.

RESULTS AND DISCUSSION

In last decade, several reports have highlighted the involvement of AdoMet as a key regulator in different cellular processes such as proliferation, cell differentiation, and apoptosis both *in vitro* and *in vivo* (48, 50-75).

Clinical outcome of LSCC remains discouraging despite intensive surgery, radiotherapy and chemotherapy. The significant morbidity associated with current treatment modalities has led to continuing investigation of more effective and less toxic therapies.

AdoMet, in addition, is an approved nutritional supplement thus lending itself to be used for therapeutic purposes without the common contraindications of chemotherapy drugs.

Despite emerging data from literature on the anti-proliferative and anti-metastatic effects exerted by AdoMet in a variety of cancer cells are accumulating, today, only few data are available on the molecular mechanisms underlying the anticancer effect of AdoMet in LSCC.

Moreover, recent studies have reported that the action of AdoMet can also be mediated by a new class of small non-coding RNAs, which play a significant role in the pathogenesis of larynx cancer (118), where they can become important therapeutic targets.

The goals of the present research is to get insight into the molecular mechanisms underlying the antitumor activity of AdoMet in LSCC treatment, the possible use of AdoMet in combination with common drugs used in laryngeal therapy and the study of the regulation of microRNAs by the sulfonium compound in laryngeal cancer.

AdoMet induces JHU-SCC-011 laryngeal cancer cell growth inhibition

We firstly evaluated the antiproliferative activity of AdoMet on laryngeal cancer JHU-SCC-011 cells, using concentrations of AdoMet ranging from 3μM to 1mM, for different times of treatment.

Cell viability was evaluated by3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and by direct cell number counting as explained in ‘‘Materials and Methods’’ section. As reported in Figure 11a, the growth inhibitory effects induced by AdoMet were dose and time-dependent with an IC50 value of 500 μM and 300 μM after 48 and 72 hours, respectively (Fig. 11a).

Thereafter, to better analyze the antiproliferative effect of AdoMet concentrations, we carried out time-course experiments with 200 and 300 μM AdoMet for 24, 48, and 72 hours

and evaluated cell proliferation by direct cell number counting. As shown in Figure 11bAdoMetcaused a statistically significant reduction of JHU-SCC-011cell viability ($p < 0.05$), achieving at 300 μM about 8%, 25%, and 50% growth inhibition after 24, 48, and 72 hours, respectively.

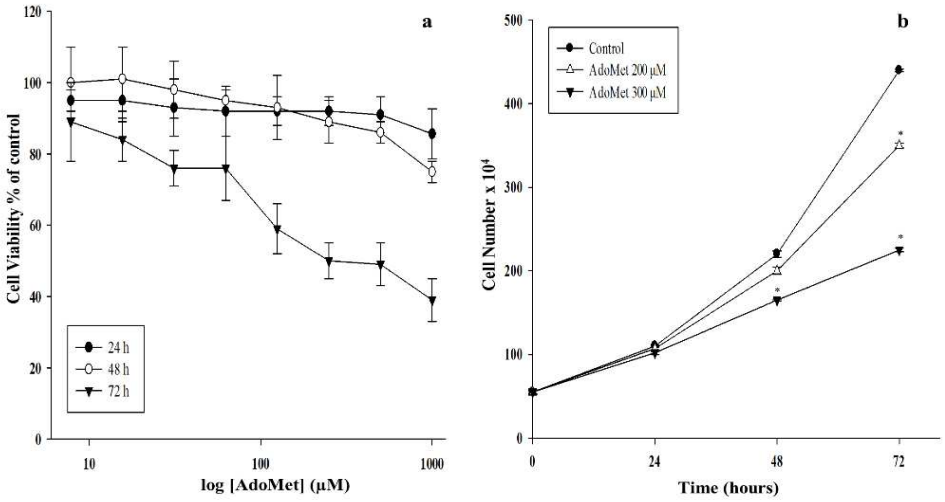

Fig. 11 - **Effects of AdoMet on JHU-SCC-011 cell growth. a:**dose-response. JHU-SCC-011 cells viability was assessed by MTT assay after exposure to different concentrations of AdoMet (from 3 μM to 1 mM) for 24, 48 and 72 hours. **b:** time-course. JHU-SCC-011 cells were treated or not (control) with AdoMet 200 and 300 μM for 24, 48 and 72 hours and cell number was measured. Data represented the average of three independent experiments. The means and SD are shown.* $P < 0.05$ versus control untreated cells.

Our results demonstrated that AdoMet inhibits the proliferation of LSSC cells and are consistent with our reported data about the effects of the sulfonium compound on other types of human cancer cells (51-76) and on HNSCC (119).

AdoMet triggers apoptotic cell death

In order to analyze the mechanism by which AdoMet exerts its antiproliferative action in JHU-SCC-011 cells we evaluated apoptosis occurrence.

Apoptosis or Programmed Cell Death is a regulated pathway that plays a key role in process of cellular self-destruction during development, tissue homeostasis, and immune system function. It is characterized by specific morphological and biochemical properties.

Morphologically, apoptosis involves a series of structural changes in dying cells such as condensation of the cytoplasm and nucleus, degradation of cytoskeletal filaments and cellular fragmentation into membrane apoptotic bodies (120). Biochemically, apoptosis is

characterized by three main types of biochemical changes: 1) activation of caspases, 2) DNA and protein breakdown, 3) membrane changes and recognition by phagocytic cells.

The apoptotic cells express on their surface markers, like phosphatidylserine that flag up the cell for phagocytic recognition by adjacent cells or for clearance through phagocytes. This leads to the release of pro-inflammatory cellular components (120-121), leading later to the fragmentation of DNA by endonucleases. Ultimately, the pathway converge in the execution phase by activating proteases called 'caspases'. These proteases are the final executioners of cell death by proteolytic cleavage of different vital cellular substrates including nuclear lamins, DNA repair enzymes such as PARP (polyADP-ribose-polymerase) and cytoskeletal proteins. They also activate DNAase, which further degrade nuclear DNA (121-122).

Defects in the programmed suicide machinery of cell characterize all tumors and are relevant for malignant transformation, tumor formation, progression and therapeutic outcome (120). These findings highlight the importance of activating this mechanism for cancer treatment.

JHU-SCC-011 cells were treated with 300 μM AdoMet, and apoptotic process was evaluated after48, and 72 hours by FACS analysis after double labeling with Annexin V-fluorescein isothiocyanate (FITC) and PI. JHU-SCC-011 reaching only 4% of apoptosis at 48 hours (data not shown) while about 19% of cell population underwent to apoptosis after 72 hours of treatment with AdoMet (Fig. 12a).

In conclusion, AdoMet showed a significant pro-apoptotic effect on JHU-SCC-011 cells.

To explore the mechanism of apoptosis-mediated cell death, we next assessed the effects of sulfonium compound on caspase activation cascade (Fig. 12b). In details, JHU-SCC-011 cells were treated with 200 and 300 μM AdoMet for different times and caspase fragmentation was analyzed by Western blotting.

As reported in Figure 12bAdoMet treatment strongly reduced the expression of full length caspase 9, initiator of apoptosis, and caspase 6 and caspase 7, downstream caspases, with a concomitant decrease of full length PARP-1, a known target for apoptosis-associated caspase cleavage (122-123).

The balance of the expression of anti- and pro-apoptotic members of the Bcl-2 gene family is one of the major mechanisms that regulate the apoptotic process in mammalian cells (124). The Bcl-2 family consists of numerous high-homologous isoforms that either inhibit

(Bcl-2, Bcl-x_L, Mcl-1 and Bcl-W) or promote (Bax, Bad, Bak, Bcl-x_S and Bok) apoptosis.

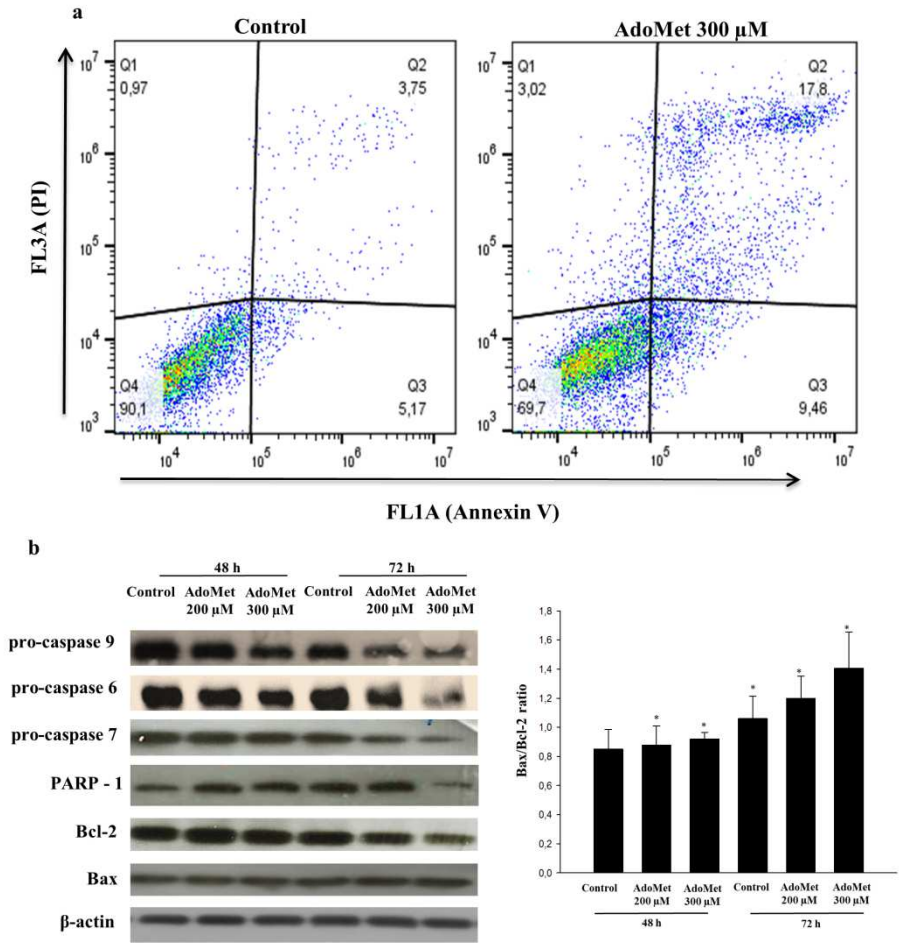

Fig. 12 - Apoptosis assessment in JHU-SCC-011cell lines treated with AdoMet. JHU-SCC-011 cells were cultured in medium supplemented or not (control) with AdoMet 300 µM for 48 and 72 hours. then apoptosis was evaluated. **a:**Representative dot plots of both Annexin V-FITC and PI-stained. The percentage of cells is reported in the quadrants. Viable cells, lower left (Q4); non-viable necrotic cells, upper left (Q1); early apoptotic cells, lower right (Q3); late apoptotic cells, upper right (Q2).**b:**Western blotting assay. 20 µg of protein extracts were immunoblotted with antibodies against the indicated proteins. For the equal loading of protein in the lanes, the expression of the house-keeping protein β-actin was used as a standard. Graphs show the densitometric intensity of Bax/Bcl-2 bands ratio. The intensities of signals were expressed as arbitrary unit. * $P < 0.05$ versus control untreated cells. The image is representative of three immunoblotting analysis obtained from at least three indipendent experiments.

Most Bcl-2 family members are expressed simultaneously in the cell, and the balance of pro-and anti-apoptotic forms is important to cell survival or commitment to cell death (124).

Therefore, we have also evaluated the levels of two mitochondria-associated modulators of apoptosis, the pro-apoptotic Bax and the anti-apoptotic Bcl-2 proteins, to determine whether AdoMet-induced apoptosis was associated to the modulation of members of this protein family.

The treatment with AdoMet induced the increased expression of pro-apoptotic Bax while the levels of anti-apoptotic Bcl-2 was strongly decreased. It has to be noted that Bcl-2 serves as the mitochondrial gate-keeper and is frequently overexpressed in tumors where it plays a critical role in chemo- and radio-resistance(124-125).

All the data above indicated that AdoMet could efficiently induce apoptosis in JHU-SCC-011 laryngeal cancer cells by activating caspase-dependent mechanism paralleled by increased Bax/Bcl-2 ratio (Fig.12).

Autophagy assessment in JHU-SCC-011 cell lines treated with AdoMet

In order to assess cell death mechanisms different from apoptosis induced by AdoMet on JHU-SCC-011 cells, we evaluated autophagy occurrence.

Autophagy is an evolutionally conserved stress response, characterized by the sequestration of cytoplasmic material within autophagosomes and subsequent degradation by lysosomes. The autophagy begins with the assembly of protein complexes on isolated membrane to initiate the formation of autophagosome, followed by its nucleation, elongation and maturation. Fusion of autophagosomes with lysosomes then leads to degradation of the cargo (126).

All cells show a low basal level of autophagy, which is necessary to perform homeostatic functions; however, this autophagy is massively upregulated during starvation or trophic factor withdrawal, architectural changes (developmental processes) or the turnover of damaged cytoplasmic organelles (during oxidative stress or infections). In fact, the degradation of intracellular organelles and proteins provides a source of amino acids, nucleotides and fatty acids and energy to maintain cell viability through the bulk degradation of cytoplasmic material.

One controversially discussed question is whether autophagy is a death execution mechanism or whether autophagic activity is a survival strategy aimed to prevent cell death (127).

To evaluate the autophagic occurrence we performed fluorescence microscopy and a quantitative analysis by FACS using the vital dye LysoTracker Red (LTR), a fluorescent

probe for labeling and tracking of acidic organelles, including autophagosome and autolysosome structures, in living cells (128). Figure 13a and b show both qualitative and quantitative analyses demonstrating a consistent increase in autophagic flux after 200 and 300 µM AdoMet, compared to untreated cells, especially after 24 hours.

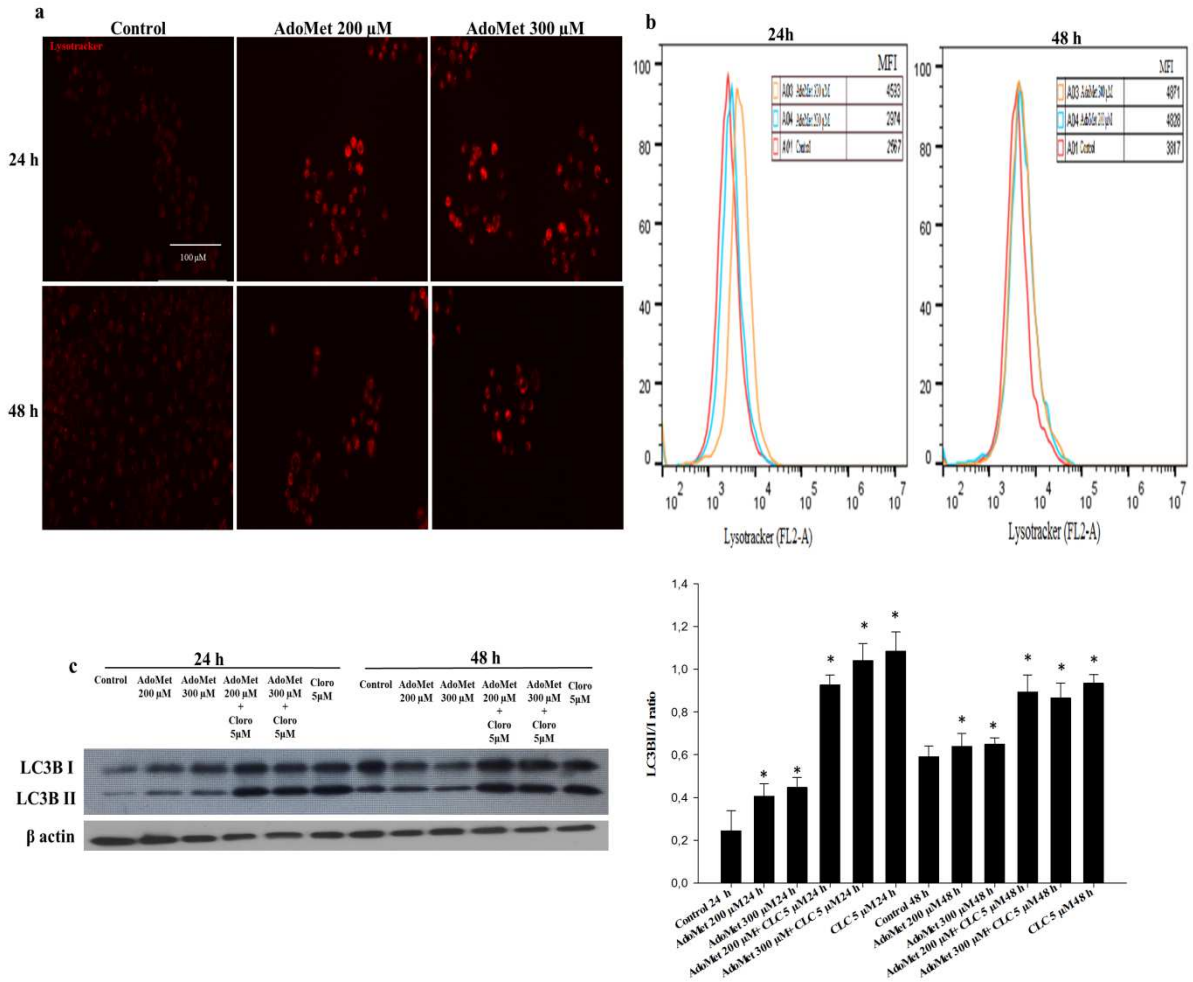

Fig. 13 - Effects of AdoMet on autophagy process.JHU-SCC-011 cells were cultured in medium supplemented or not (control) with AdoMet 200 and 300 µM for 24 and 48 hours. **a:** cells were incubated with LTR and analyzed by fluorescence microscopy. **b:** cells were incubated with LTR and analyzed by flow cytometry. **c:** Western blotting assay for the expression of autophagy-related markers LC3B; graph shows the densitometric intensity of LC3B-II/I bands ratio. For the equal loading of protein in the lanes, β-actin was used. The experiments were repeated at least three times and always gave similar results.

To confirm the induction of the autophagic process by AdoMet we detected the levels of LC3B, a main marker used to monitor autophagy, in presence of AdoMet alone or in combination with chloroquine (CLC), the main lysosomal protease inhibitor. LC3B is a

protein normally placed in the cytosol (LC3B-I) but upon induction of autophagy it becomes lipidated, cleaved to form LC3B-II, and embedded in autophagosomal membranes (LC3B-II) (129-130). Thus, the amount of LC3B-II is correlated with the extent of autophagosome formation but not indicate the total autophagic flux. This flux is more accurately represented by differences in the amount of LC3B-II between samples in the presence and absence of lysosomal protease inhibitors. Figure 13c shows that after 24 and 48 hours of treatment, LC3B-II/I protein ratio was significantly increased above all at 24 hours, and that the further accumulation of LC3B-II in presence of AdoMet and CLC is due to the enhancement of autophagic flux rather than the inhibition of autophagosome degradation.

According to the obtained results, we suggest that AdoMet is able to induce autophagy in JHU-SCC-011 cells by increasing autophagic vacuoles and enhancing the levels of the autophagic marker LC3B-II.

AdoMet induces endoplasmic reticulum stress and unfolded protein response in JHU-SCC-011 cell line

In order to confirm the underlying mechanisms of AdoMet-induced apoptotic cell death in LSCC, we evaluated its effect on endoplasmic reticulum(ER)-stress and the related unfolded protein response (UPR) on laryngeal cancer cell line JHU-SCC-011.

ER-stress plays an important role in drug-induced apoptosis thus resulting a potential target with significant clinical importance in chemotherapy (131-133). The ER is the subcellular entrance for a number of secretory and structural proteins as well as the site of biosynthesis for steroids, cholesterol, and other lipids. It provides a unique environment for correct protein folding, assembly, and disulfide bond formation, leading to production of functional, mature proteins.

Several physiological and pathological stimuli, among them cytotoxic compounds administration, deregulate normal ER activity resulting in a stress condition termed “ER-stress” characterized by misfolded proteins accumulation inside the ER lumen.

It is known that homeostasis in the ER is maintained via a coordinated adaptive program, so-called the UPR.

In response to ER-stress, the UPR initially engages adaptive outputs that decrease the load of unfolded proteins in an effort to restore ER homeostasis. However, when ER stress cannot be reversed, the UPR in mammals assembles into a signaling platform that translate pro-inflammatory and pro-death signals to hasten cell death.

Multiple studies have clarified the link between ER-stress and cancer, and particularly the involvement of the UPR. The UPR seems to adjust the microenvironment of cancer and, as such, is one of resistance mechanisms against cancer therapy (131-133).

The cells were treated with 200 and 300 μM AdoMet for 48 and 72 hours. The shape of treated cells was then analyzed by fluorescence staining using ER-Tracker, a vital dye which accumulates into ER and tunicamycin, a known inducer of ER-stress, was used as positive control. AdoMet increased fluorescence of cells compared to untreated cells (Fig. 14a), and caused morphological changes of the ER providing a first indication that AdoMet was able to trigger ER-stress in JHU-SCC-011 cells.

To confirm the effectiveness of AdoMet as an ER-stress inducer, we evaluated the activation of ER-stress markers, and their downstream signaling molecules. The quantitative analysis obtained by qRT-PCR experiments reported in Figure 14b showed that, the treatment with 200 and 300 μM AdoMet induces an evident increase of CCAAT-enhancer-binding protein homologous protein (CHOP), unspliced x-box binding protein 1 (uXBP1) and spliced x-box binding protein 1 (sXBP1) levels, especially after 72 hours of treatment. Furthermore, we have confirmed the increased expression of CHOP induced by AdoMet both at transcriptional and translational levels, as shown in Western blotting analysis reported in Figure 14c.

The identification of numerous points of cross-talk between the UPR and mitogen-activated protein kinase (MAPK) signaling pathways has contribute to a better understanding of the consequences of ER-stress. Indeed, the MAPK signaling network is known to regulate cell cycle progression and cell survival or death responses following a variety of stresses (134). Therefore, we analyzed the activation of C-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2), and mitogen-activated protein kinases 11 (p38) by Western blotting (Figure 14c) whose phosphorylation and so activation was increased by AdoMet treatment.

DUSP-1 is a dual-specificity phosphatase that selectively dephosphorylates MAPKs and is the main phosphatase that inactivates JNK (135). As a result of DUSP-1 increase, JNK activation would be inhibited, which consequently protects tumor cells from JNK-induced apoptosis (135). Based on these considerations, we examined DUSP-1 protein level in JHU-SCC-011 cells in response to AdoMet. Western blotting analysis evidenced that AdoMet remarkably decreased the level of DUSP-1 in concentration-dependent manner (Fig.14c).

Altogether these data indicate that the activation of ER-stress represents a possible mechanism underlying the pro-apoptotic effect of AdoMet.

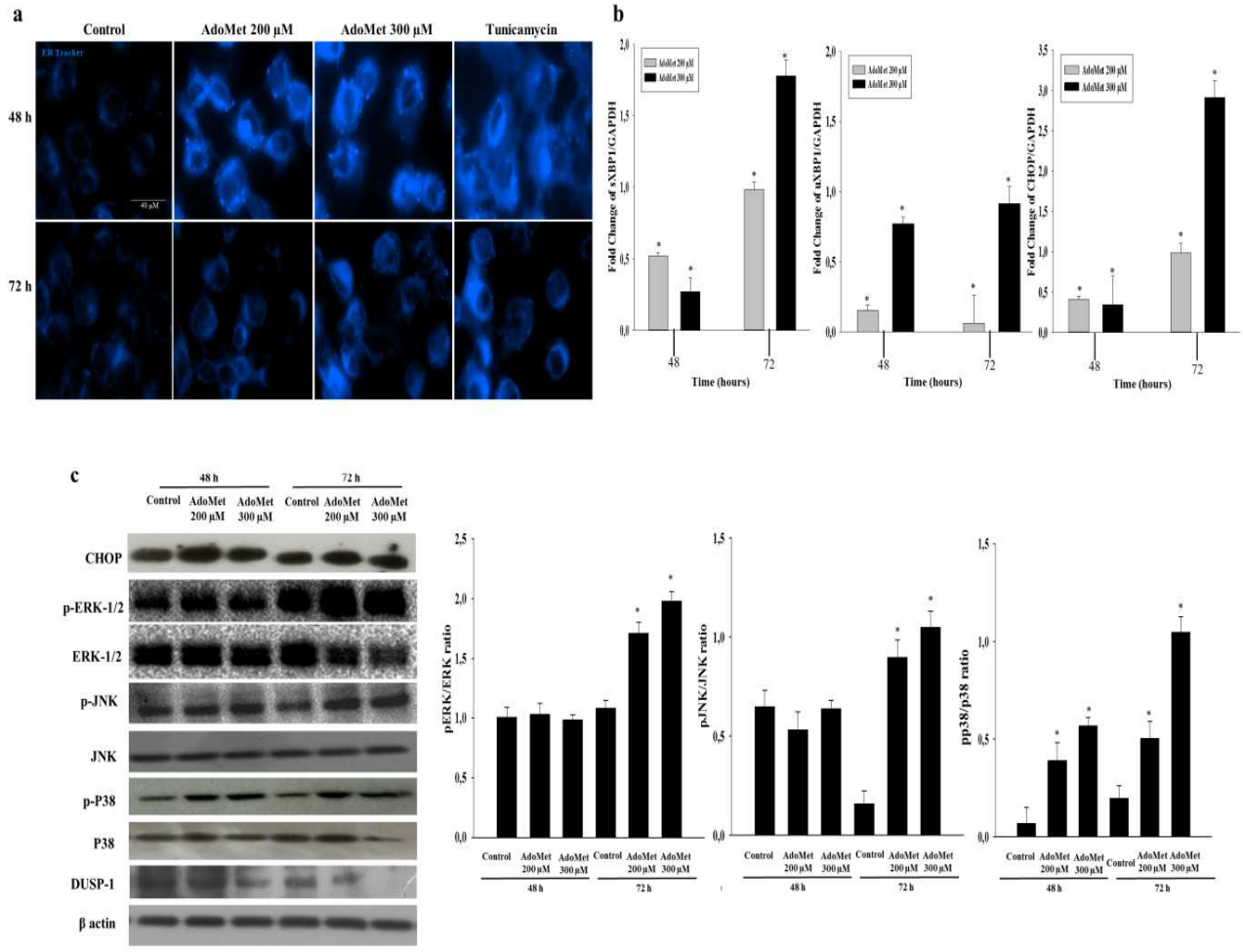

Fig. 14 - Effects of AdoMet on the UPR under ER-stress. JHU-SCC-011 cells were treated or not treated (control) with 200 and 300 µM AdoMet for 48 and 72 hours. **a:** cells were incubated with ER-tracker and analyzed by fluorescence microscopy. Tunicamycin was used as positive control. The experiments were repeated at least three times and always gave similar results. **b:** total-RNA was extracted and cDNA was synthesized for qRT-PCR, in order to investigate transcription of several key proteins involved in ER-stress. The graphs showed the transcriptional levels of uXBP1, sXBP1 and CHOP compared to untreated cells. Data represent the average of three independent experiments. The means and SD are shown.* P< 0.05 versus control untreated cells. **c:** Western blotting assay for the expression of CHOP and DUSP-1 and activation of ERK 1/2, JNK and p38. For the equal loading of protein in the lanes, β-actin was used.

AdoMet promotes ROS generation in JHU-SCC-011 cancer cell line

Radical oxygen species (ROS) are normal by-products of various cellular processes, such as signal transduction, mitochondrial metabolism, DNA repair, and protein folding (136-138). Therefore, the oxidative stress response plays a significant role in keeping the balance between prosurvival and pro-apoptotic signaling pathways (136-138). Varieties of pro-apoptotic signaling pathways can be stimulated in an uncontrolled high level of ROS status, such as mitochondrial dysfunction, ER-stress and DNA damage. Recently, it has been reported that ROS generation is partly linked to ER-stress activation (137). ER-stress increases oxidative load and can cause oxidative stress depending on the severity of ER-stress (137). On the other hand, excess accumulation of ROS in different cellular compartments, especially the mitochondria, can induce the UPR (137). These findings indicate that there is a mutual relationship between ER stress and ROS accumulation.

To better understand the mechanism of AdoMet action, here we have evaluated for the first time, the levels of ROS in cells treated with 200 and 300 μM of AdoMet after 48 and 72 hours of treatment. We performed a quantitative analysis by FACS using dichloro-dihydro-fluorescein diacetate (DCFH-DA) as a substrate. DCFH-DA passes the cell membrane where it is trapped after being deacetylated by intracellular esterases. DCFH can be oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS.

The levels of ROS increase after AdoMet treatment, above of all at 72 hours as shown in Figure 15.

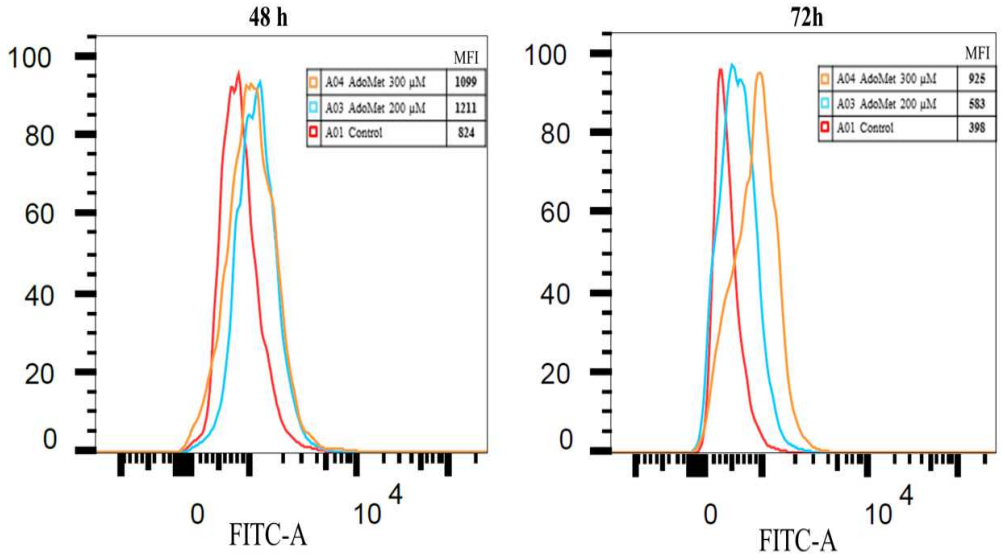

Fig. 15 - AdoMet increases ROS accumulation in laryngeal cancer cells.JHU-SCC-011 cells were treated or not treated (control) with 200 and 300 µM AdoMet for 48 and 72 hours. Cells were incubated with DCFH-DA and analyzed by FACS analysis. The experiments were repeated at least three times and always gave similar results.Data represent the average of three independent experiments. The means and SD are shown.* P< 0.05 versus control untreated cells.

Combination of AdoMet and cDDP sensitizes JHU-SCC-011 cells to the antiproliferative effects of cDDP

cDDP has in widespread use to treat several forms of cancer, mostly HNSC, however, resistance to this drug remains a major obstacle in chemotherapy (80-81). It is well established that cDDP produces significant detrimental side-effects and often the occurrence of drug resistance in cancer cells (81) results in the failure of chemiotherapy.

Previously, my research group has showed that AdoMet is able to potentiate the cytotoxic effects of cDDP in oral cancer cell line (119). In fact, we have found a synergistic increase in apoptotic occurrence with 200 µM AdoMet/0.18 µM cDDP combination.

Therefore, we have evaluated the apoptotic occurrence of the same combination after 72 hours by FACS analysis and Western blotting, also in this HNSCC cell line. As shown in Figure 16a, we have found an increase of apoptotic population in cells treated with 200 µM AdoMet/0.18 µM cDDP combination.

In order to confirm the synergistic apoptotic effect induced by AdoMet/cDDP combination we have analyzed the expression level of key enzymes involved in the apoptotic

cascade, such as pro-caspase 6, pro-caspase 9, PARP-1, Bax and Bcl-2 proteins and the more MAPK involved in cDDP response i.e. JNK and ERK1/2. As shown in Figure 16b, AdoMet induced only a slight change in the levels of apoptotic proteins, while no significant changes were observed with cDDP. On the contrary, the AdoMet/cDDP combination strongly decreased the levels of uncleaved isoforms of caspase 9, caspase 6 and of their target PARP-1 with a concomitant increase of Bax/Bcl-2, pJNK/JNK and pERK/ERK1/2 ratio (Fig. 16b).

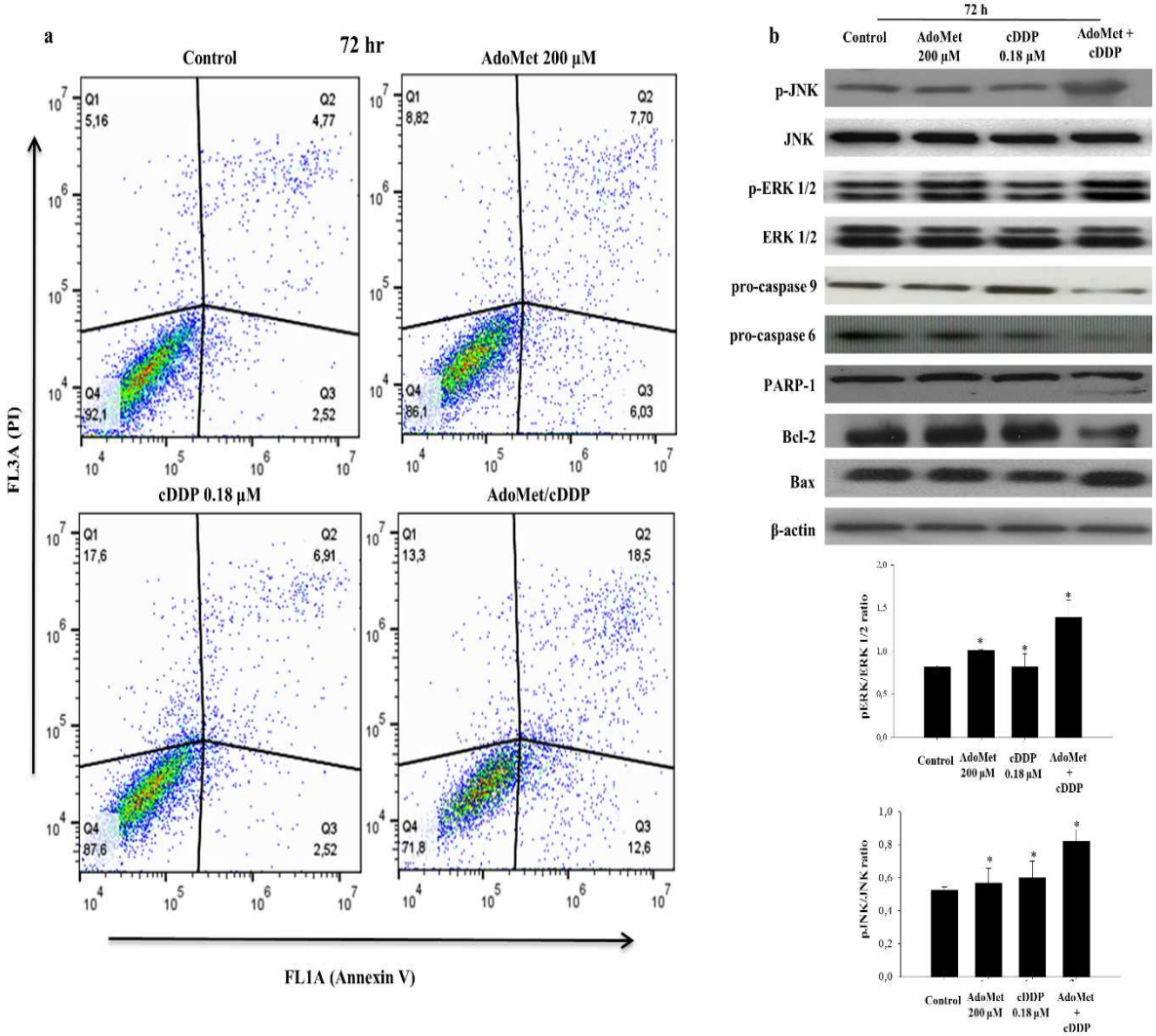

Fig. 16 -Effects of AdoMet and cDDP on apoptotic occurrence in JHU-SCC-011. JHU-SCC-011 cells were treated alone or in combination with AdoMet 200 µM and cDDP 0.18 µM for 72 hours **a:** Representative dot plots of both Annexin V-FITC and PI-stained. **b:** Western blotting assay. For the equal loading of protein in the lanes, the expression of the house-keeping protein β-actin was used as a standard. Histograms show the densitometric intensity of pERK/ERK1/2, pJNK/JNK bands ratio. The intensities of signals were expressed as arbitrary unit. * $P < 0.05$ versus control untreated cells. The image is representative of three immunoblotting analysis obtained from at least three indipendent experiments.

In conclusion, our results indicate that the combination of AdoMet and cDDP, through the activation of similar signaling pathway, enhances apoptotic cell death.

MiRNA extraction, analysis and validation by qRT-PCR

MiRNA dysregulation is common in various types of cancer and they are known to be involved in the regulation of different cellular processes including differentiation, proliferation and apoptosis (107-114). To obtain new information on the molecular mechanisms underlying AdoMet anticancer activity we have carried out miRNAs expression profiling of JHU-SCC-011 cells treated with 300 μM of AdoMet for 72 hours using a 384-well TaqMan Array CARD.

Fifteen miRNAs were differentially expressed in AdoMet-treated cells if compared to control samples. The fold change is graphically represented in Figure 17.

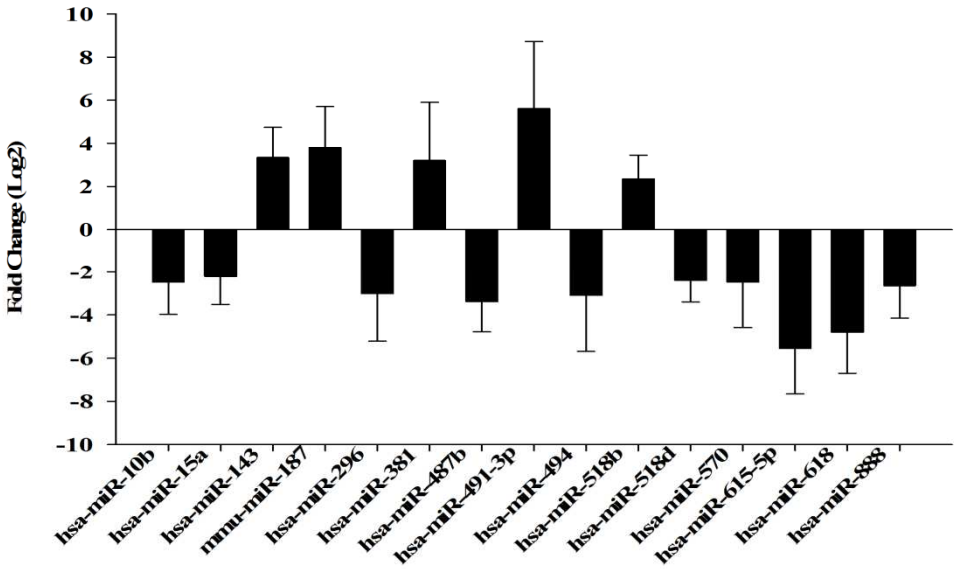

Fig. 17 - MiRNA expression pattern in JHU-SCC-011 cells treated with AdoMet

Among them, we have focused the next investigations on miRNAs that were previously reported to be most involved in the regulation of the main pathways of proliferation and cell death, such as miR-187, miR-491-3p, miR-618 and mir-888-5p.

Of these miRNAs regulated by AdoMet, only one was validated through the qRT-PCR analysis after 72 hours of 300 μM AdoMet treatment, compared to untreated, as shown in

Table 1. MiRNA-888-5p appeared remarkably down-regulated, in TaqMan Array CARD and in qRT-PCR, respectively of -2.65 and -7.63 times.

MiR-888, a newly emerging miRNA, was reported to be implicated in the tumorigenesis of endometrial cancer, prostate cancer, and breast cancer (139-142).

Table 1: Comparison of fold changes seen in microarrays and by qRT-PCR

MiRNAs	Fold-Changes (Log2)	
	Microarray	qRT-PCR
hsa-miR-888	-2,65	-7,63
mmu-miR-187	3,80	-
hsa-miR-491-3p	5,62	-
hsa-miR-618	-4,8	-

MiR-888-5p inhibitor enhances the antitumor effects of AdoMet in JHU-SCC-011 cell line.

To investigate the biological significance of miR-888-5p down-regulation in AdoMet-treated LSCC cells, we performed transfection experiments with miR-888-5p inhibitor in JHU-SCC-011 cells, and then, the modulation of apoptosis, autophagy and ROS production were analyzed by FACS analysis.

It has emerged that miRNAs have key roles in the regulation of distinct biological processes in mammals, including development, differentiation, apoptosis, survival, senescence, and metabolism (107-114).

The interest in the study of therapeutic strategies focus on miRNAs is linked to the fact that by intervening therapeutically on a single molecule it is possible to obtain multiple effects on the gene expression of the cell. MiRNAs with tumor suppressor function, which are generally under-expressed in tumors, can be used as therapeutic molecules to cause cancer cells to die. On the contrary, to inhibit the expression of oncomiRs it is possible to use molecules able to bind stably the miRNA, subtracting it from its biological function (89-90)

MiRNA inhibitor are single-stranded, antisense oligonucleotides designed to inhibit miRNA function. The perfect complementarity of the sequence allows the antisense oligonucleotide to bind to the miRNA and interfere with its function. Mimic miRNAs are short double-stranded oligonucleotides in which one strand is identical to the mature miRNA

sequence (guiding strand) and the other strand is complementary or partially complementary and complexed with the mature miRNA sequence (passenger filament)in order to increase the level of miRNA of interest and to block targeted gene expression more efficiently (88-89).

The results revealed that the transfection with 100 nM miR-888-5p inhibitor induced about 17% apoptosis in JHU-SCC-011, while in agreement with our previous results about 19% of cell population underwent to apoptosis after treatment for 72 hours with 300 μM AdoMet, as shown in Figure 18a. Moreover, the combination of AdoMet and miR-888-5p inhibitor strongly increased apoptotic cell death of 50% in JHU-SCC-011 (Fig.18a).

The pro-apoptotic effect was mediated by caspase dependent process demonstrated by Western blotting analysis of pro-caspase and PARP-1 decrease (Fig. 18b).

In addition, combination treatment with AdoMet and miR-888-5p inhibitor induced an increase in the autophagy process and ROS production.

As already mentioned, AdoMet strongly induces the activation of the autophagic flux as well as an increase in ROS production.

To investigate the possible implication of miR-888-5p in the induction of these pathways by AdoMet, cells were transfected for 72 hours with miR-888-5p inhibitor, in presence or not of AdoMet.

The autophagic process was performed by fluorescence microscopy, FACS analysis and LC3BII/I ratio by Western blotting, while an increase of ROS production was take over by flow cytometry (Fig19).

The both processes were increased by the combination treatment more than with AdoMet or miR-888-5p inhibitor alone, demonstrating that miR-888-5p is involved in the activation of these pathways.

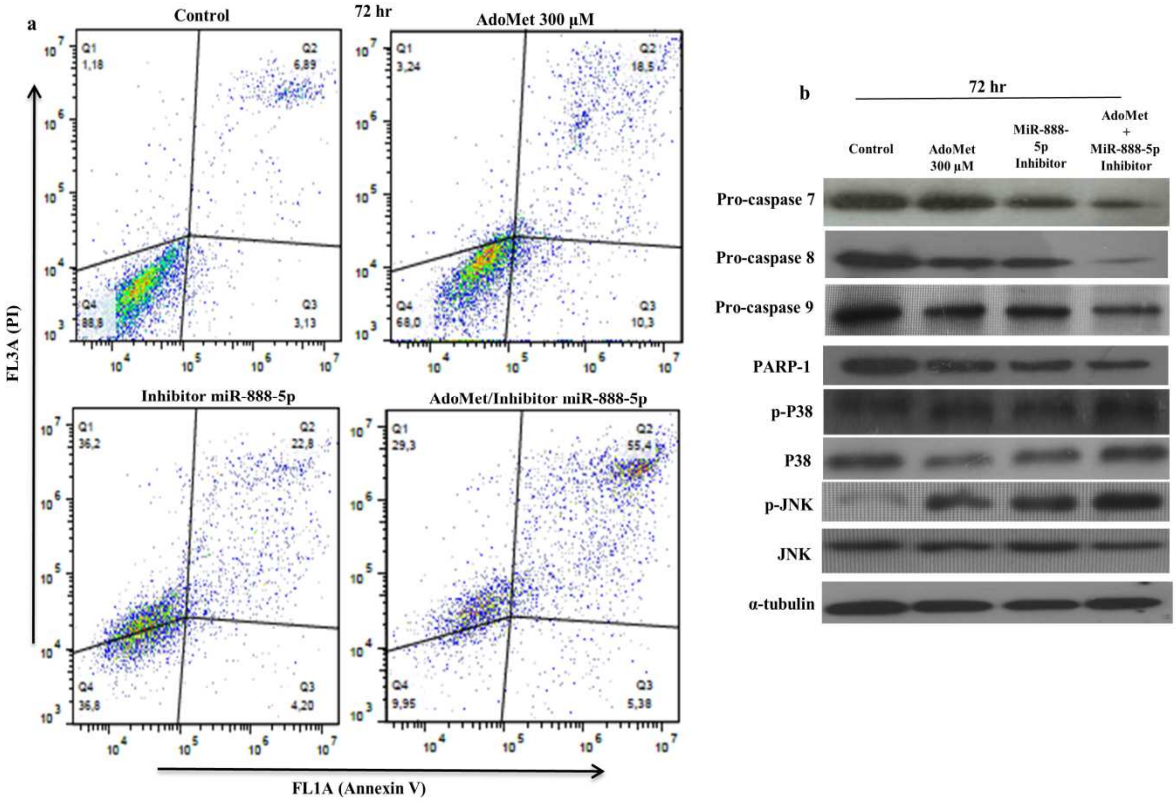

Fig. 18 - Effects of AdoMet and miR-486-5p mimic or inhibitor combination on autophagy process. Cells were treated or not treated (control) with 300 µM AdoMet and/or miR-888-5p inhibitor for 72 hours. **a:** Apoptosis was evaluated by FACS analysis. Representative dot plots of both Annexin V-FITC and PI-stained. **b:** Western blotting assay of JHU-SCC-011 cell extract for the expression of pro-caspase 7, pro-caspase 8, pro-caspase 9, PARP-1, JNK, pJNK, P38, pP38. For the equal loading of protein in the lanes, the expression of the house-keeping protein α-tubulin was used as a standard. The intensities of signals were expressed as arbitrary unit. * $P < 0.05$ versus control untreated cells. The image is representative of three immunoblotting analysis obtained from at least three indipendent experiments.

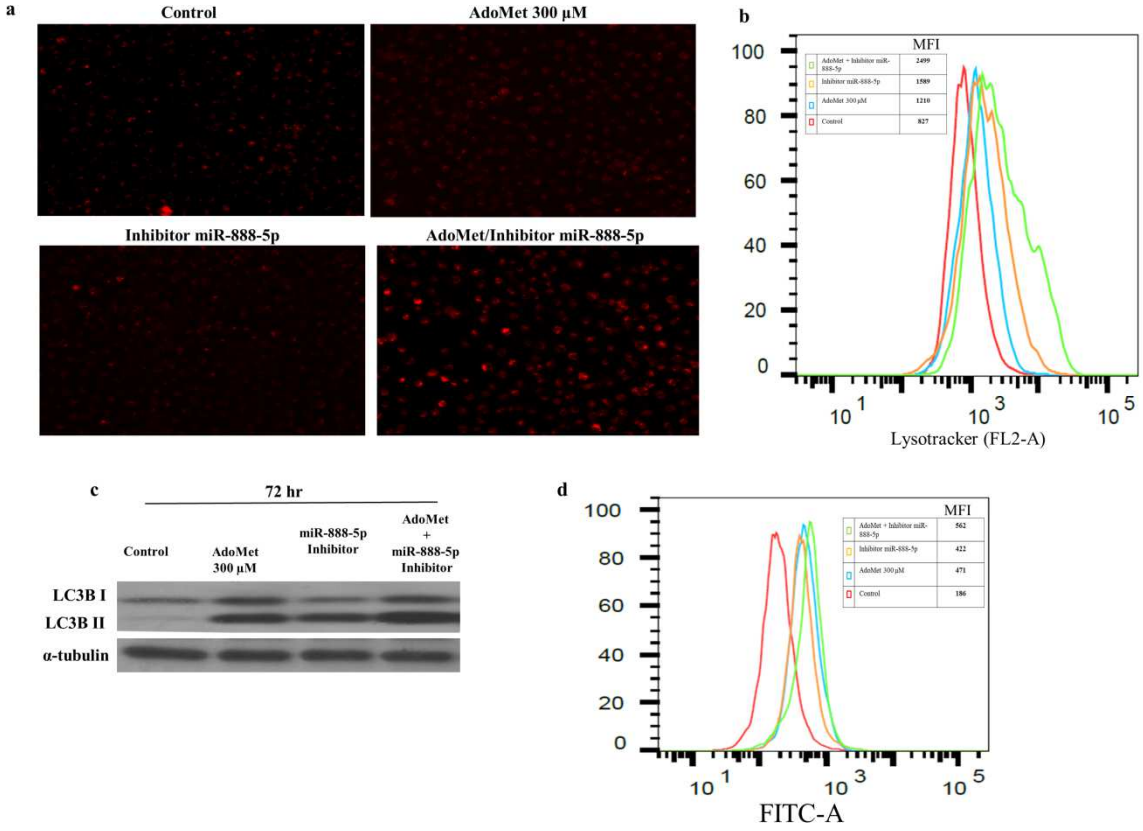

Fig.19 -Effects of AdoMet and miR-888-5p inhibitor combination on autophagy process and ROS production. Cells were not treated (control) or treated with 300 µM AdoMet and/or miR-888-5p inhibitor for 72 hours. **a:** cells were incubated with LTR and analyzed by fluorescence microscopy. **b:** cells were incubated with LTR and analyzed by flow cytometry. **c:** Western blot assay for the expression of autophagy-related markers LC3B. For the equal loading of protein in the lanes, α-tubulin was used. **d:** cells were incubated with DCFH-DA and analyzed by FACS analysis. The experiments were repeated at least three times and always gave similar results.

In conclusion, these data evidence that combination of AdoMet with miR-888-5p inhibitor potentiates the signaling pathway activated by AdoMet treatment, providing a preliminary indication that AdoMet antitumor effects could be mediated by its regulation on this miRNA.

MKK4 is a potential target of miR-888-5p

To identify potential mRNA targets of miR-888-5p, we performed a miRNA-mRNA integration analysis by using the TargetScan and miR-DB microRNA target prediction software. The analysis identified 826 potential miR-888-5p targets, based on their sequence

complementarity, including mixed lineage kinase domain like pseudokinase (MLKL), FAS, receptor-interacting protein kinase 3 (RIP3) and mitogen-activated protein (MAP) kinase kinase 4 (MKK4 or MAP2K4).

Among them, we focused our attention on MKK4, that is a component of stress activated MAP kinase signaling. The alignment of miRNA-888-5p/MKK4 is shown in Figure 20.

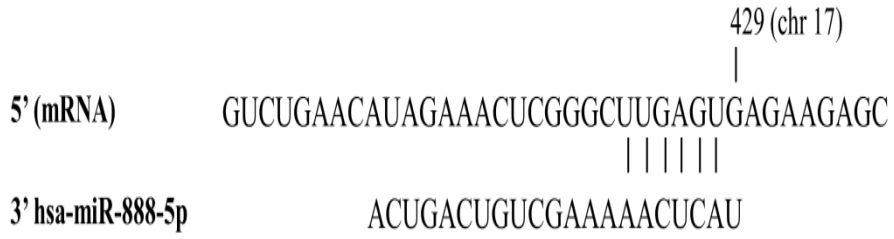

Fig. 20 - Alignment of miR-888-5p with MKK4 3'UTR

The MAPKs pathways are evolutionarily conserved among eukaryotes and feature a triple kinase cascade comprised of the MAPK which is phosphorylated and activated by a MAP kinase kinase (MKK), which itself is phosphorylated and activated by a MAP kinase kinase kinase (MKKK).

Recently, a number of studies have supported a role for MKK4 in regulating steps in the development of cancers and in the suppression of metastasis (143-144). MMK4 has been identified a tumor suppressor and its new role is supported by the fact that theMKK4gene is mutated at a frequency of 5% in different cancer types.

In vivo experiments show that mice with a targeted deletion of the MKK4gene die during embryogenesis providing clear evidence that MKK4 has distinct functions in cells that cannot be complemented by other MKKs.

MKK4 is unique among the mammalian MKK family in its ability to phosphorylate and activate two MAP kinase groups: JNK and p38 that act as tumor suppressors through activation of apoptosis (145).

The JNK pathway is a well characterized player of apoptosis (146), while the role of p38 in cancer has received less attention, although there is an increasing body of evidence suggesting that it participates in tumor suppression (147). JNK is able to regulate differently the pro-apoptotic and anti-apoptotic Bcl2 family members (148-153). JNK phosphorylates

and inactivates the anti-apoptotic Bcl2, Bcl-XL and Mcl-1, while it is able to phosphorylate and activate the pro-apoptotic Bax (154-156).

Activated p38 regulates the activity of a wide range of protein kinases transcription factors (like CHOP and p53) and some other proteins, which then further regulate the activity of their targets. The mechanisms by which p38 contributes to an enhanced pro-apoptotic response include the phosphorylation and translocation of proteins from the Bcl-2 family, which leads to the release of cytochrome c from the mitochondria (154-156) the transforming growth factor-β-induced activation of caspase 8 (157-158) as well as the regulation of membrane blebbing and nuclear condensation (159).

Moreover, apoptosis induced by chemotherapy and genotoxic drugs is mediated by the activation of MAPKs signaling. Sustained activation of JNK/p38 MAPK pathways by cDDP has resulted one of the major causes for the increased chemosensitivity to cDDP in inducing apoptosis in ovarian carcinoma cells (160, 161). Previous studies also showed that inhibition of either JNK or p38 kinase attenuated cDDP induced apoptosis in cervical cancer cells, supporting the role of these MAPK in cDDP response (162-163).

Based on these findings in literature, MAPK research is attracting more and more attention in cancer therapy, and its targeting by regulation of MKK4 could represent a good strategy for cancer treatment.

Therefore, additional experiments currently still ongoing, must be performed to verify that MKK4 is one of the predicted targets of miR-888-5p.

CONCLUSION

LSCC, with higly invasive and metastatic malignant behavior has the second highest incidence in all HNSCC, and despite recent improvement in oncological and surgical treatment, the prognosis remains extremely poor (164).

New advances in anticancer drug discovery using natural compounds have been made in the last years in order to improve therapeutic treatment options. Surgery, radiation, chemotherapy, targeted treatments and immunotherapy, separate or in combination are commonly used to treat laryngeal cancer. However, these treatment types may cause different side effects, and chemotherapy-based regimens appear to have reached a therapeutic plateau. Hence, effective, better-tolerated treatments are needed to address and hopefully overcome this problem.

CDDP is the most effective and widely used chemotherapeutic agents employed for treatment of solid tumors. It is a platinum-based compound that forms intra- and inter-strand adducts with DNA, and thus it is a potent inducer of cell cycle arrest and apoptosis in most cancer cell types (165). Unfortunately, many patients with these malignancies eventually relapse and become refractory (drug resistant) to chemotherapy (166-167). Cancer cells can develop cDDP resistance through changes in reduced drug uptake, increased drug inactivation and DNA adduct repair.

Some natural compounds may sensitize to conventional cytotoxic therapy, reinforce the drug effective concentration, intensify the combined effect of both administered therapeutics or exert cytotoxic effects specifically on tumor cells. Moreover, combined therapy by targeting multiple signaling pathways, uses various mechanisms to reduce the development of resistance to antitumor drugs (83-86).

In this context my PhD research is included, aimed to better understand the antitumoral activity of AdoMet in LSCC and to evaluate its possible use in synergism with cDDP.

AdoMet represents an important and naturally occurring sulfonium compound found in all mammalian cells, in which it exerts a variety of well-documented biological functions (1-5, 22).

Recently, the potential of AdoMet as antiproliferative agents has been evidenced in the literature, and growing scientific interest is focused on identifying the biological mechanisms and the signal transduction pathways related to the chemopreventive activities of this natural sulfur-nucleoside (40-41, 50-61). AdoMet, in addition, is an approved nutritional supplement

thus lending itself to be used for therapeutic purposes without the common contraindications of chemotherapy drugs.

In the present study we have investigated AdoMet anticancer activities in JHU-SCC-011 cancer cells providing experimental evidences on the underlying mechanisms.

AdoMet induced ER-stress and activated the UPR. Although activation of the ER stress response leads to adaptations that may aid in cell survival, it is well known that, under severe and prolonged ER-stress conditions where the cells fail to restore ER homeostasis, the UPR activates pathways that lead to apoptotic cell death. Therefore, ER plays a significant role in cancer cell apoptosis signaling pathway and its targeting can be a promising anticancer strategy.

We have shown that AdoMet induced ER-stress through the increase of the spliced form of XBP1 and CHOP levels. CHOP is one of the key components of the ER-stress-mediated apoptotic pathway; implicated in the regulation of processes that lead to cell proliferation, differentiation, expression, and energy metabolism of cell type-specific genes.

A widely accepted mechanism of CHOP-induced apoptosis is a different regulation of pro and anti-apoptotic protein, leading to the suppression of the pro-survival protein Bcl-2 and up-regulation of Bax (168).According to this, in our experimental model, AdoMet-induced apoptosis is paralleled by ER-stress, and increased CHOP expression and Bax/Bcl-2 ratio suggesting that apoptosis could be induced by mitochondria-dependent activation mediated by ER-stress.

Another important target downstream of ER-stress pathway includes several members of MAPKs family. The role of MAPKs has been controversial as their activation could result both in cell proliferation and cell death. It has been known that ERK and JNK induction, could be due to DNA damage, environmental and genotoxic stresses that subsequently lead to apoptosis (100). In this light, we have hypothesized that AdoMet triggered ER-stress induced apoptosis, through the increase of CHOP, and phosphorylation of JNK, ERK and p38.

Sustained activation of JNK and p38 can contribute to both necrotic and apoptotic cell death by regulating the expression of cytotoxic ligands (for example, FasL and TNF) (169) and synthesis of ROS (170). In addition, stress-activated MAPKs can regulate the intrinsic apoptosis pathway, mediated by mitochondria, by regulating members of the Bcl2 family.

Moreover, there is a mutual relationship between ER-stress and ROS accumulation. ER-stress increases oxidative load and can cause oxidative stress depending on the severity of ER-stress (171). On the other hand, excess accumulation of ROS in different cellular

compartments, especially the mitochondria, can induce the UPR (171). The treatment with AdoMet is able to increase ROS production in LSCC but further investigation, currently still ongoing, are needed to understand if AdoMet-induced ROS production is upstream or downstream of ER-stress response and also if the neutralization of ROS totally protects LSCC from the cytotoxic effects of AdoMet.

Furthermore, targeting ROS and oxidative stress can also be seen as a good strategy to eliminate cancer cells. Indeed, this treatment concept was already exemplified by various ROS-enhancing drugs (172-174).

According to this and to the findings that cDDP, as AdoMet, treatment results in activation of MAPK pathways, we decided to investigate the synergistic effect of these two compounds. It was reported that in squamous cell carcinoma of skin, cDDP leads to a persistent activation of JNK that is related to cell death by apoptotic pathway (175).

Moreover, in HeLa and A549 cells ERK activation is involved in cDDP-mediated apoptosis. In fact, downregulation of ERK signaling leads to an inhibition of cDDP induced apoptosis (176).

We have demonstrated that AdoMet sensitized oral cancer cells to the anti-cancer effects of cDDP. It is of great interest that in JHU-SCC-011 cells cDDP alone at the concentration of 0.18 μM was not able to induce a significant increase of apoptotic cells, differently from the combination of the same concentration of the two compounds. These data are consistent with our reported data about the effects of the sulfonium compound and cDDP combined treatment on HNSCC (119).

The regulation of miRNAs by natural compounds represents a promising approach in the field of chemotherapy research. A single miRNA can influence the expression of several targets genes, and miRNAs are implicated in the regulation of various diseases, including cancer. Several studies have demonstrated that miRNAs can act as potential oncogenes or as tumor suppressor genes during the progression of cancer, as well as crucial regulators in carcinogenesis and tumor progression (107).

The analysis of miRNA expression profile carried out through miRNA microfluidic array CARD, performed after 72 hours of treatment with 300 μM AdoMet, revealed the presence of different de-regulated miRNAs in cells treated with the sulfonium compound, compared to the control. Most of them have targets involved in processes related to cancer such as proliferation, cell survival and death pathways.

Of these miRNAs regulated by AdoMet, only one, miR-888-5p, was validated through the qRT-PCR analysis after 72 hours of 300 μM AdoMet treatment, compared to untreated cells.

MiR-888 has been recently identified as a potent oncomiR in process of endometrial cancer, prostate cancer, and breast cancer (139-142).

Lewis *et al.* found that overexpression of miR-888 significantly increases the proliferation and migration of prostate cancer cells (139), and Huang *et al* showed that miR-888 is a repressor of the adherens junction pathway in breast cancer (140).

Nevertheless, little is known about the roles of miR-888 in the clinical pathological correlations and biological functions in larynx tumorigenesis.

Therefore, we test how miR-888-5p regulated AdoMet related pathways. The analysis of the main AdoMet mediated processes revealed that miR-888-5p inhibitor potentiate the pro-apoptotic, pro-autophagic and pro-oxidative effects of AdoMet, leading us to think that the action of AdoMet can be mediated by its regulation on miRNA-888-5p level.

Through the use of the bioinformatic TargetScan software, we identified potential miRNA-mRNA-protein interactions. The most relevant and interesting for our studies is MKK4, an important component of stress activated MAPKs signaling.

A deep study in literature has revealed that MKK4 has as major phosphorylation targets JNK and p38 (145,148-159), both involved in the apoptosis induced by chemotherapy and genotoxic drugs, as discussed widely above. However, more experiments currently still ongoing, must be performed to verify this predicted targets.

In Figure 21 it is summarized the antitumor activities of AdoMet in LSCC.

Overall, these data suggest that AdoMet may provide a therapeutic approach for larynx cancer treatment targeting activation of MAPKs and that this mechanism, can be mediated by AdoMet inhibition of miR-888-5p expression.

In conclusion, the sulfonium compound could be used as a viable and attractive anticancer agent, resulted in increased therapeutic efficacy of chemoterapy treatment currently used for laryngeal cancer.

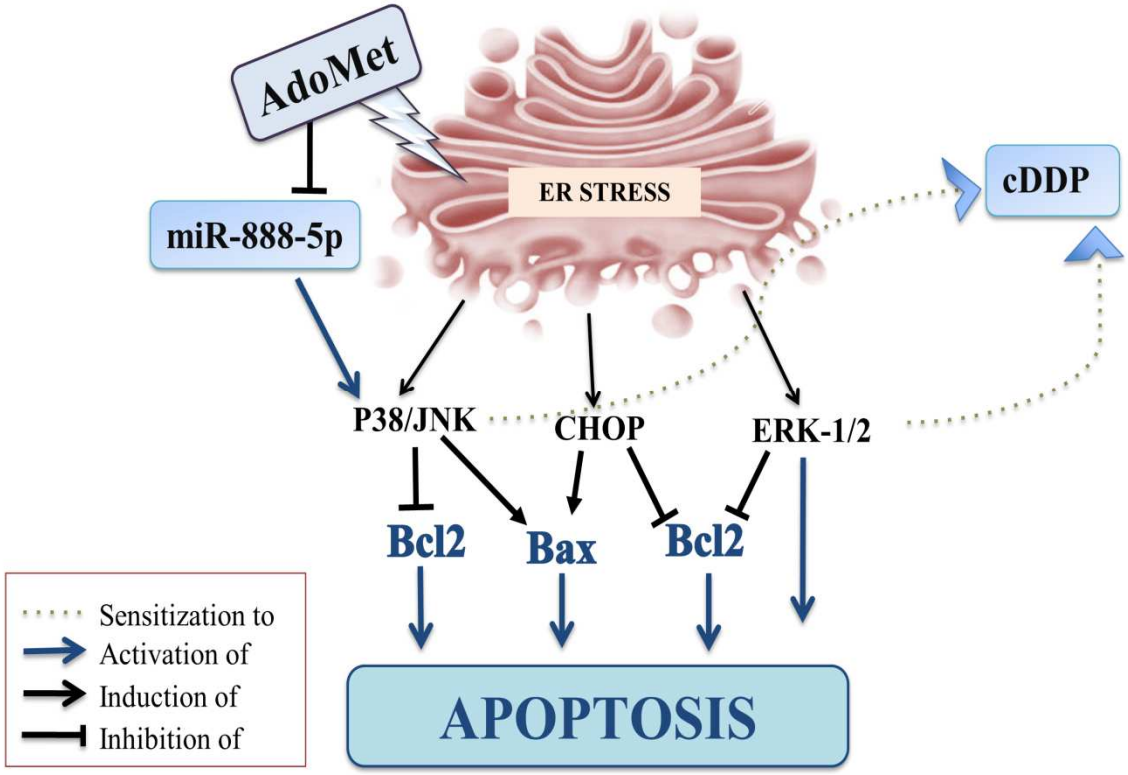

Fig. 21 - Schematic diagram summarizing the antitumor activities of AdoMet in JHU-SCC-011 cells and the proposed underlying mechanism.

MATERIALS AND METHODS

Materials

cDDP, PI , MTT, RIPA Buffer, 2′,7′-DCFH-DA and CLC were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA), fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI), phosphate-buffered saline (PBS), and trypsin-EDTA were obtained from Gibco (Grand Island, NY). Tissue culture dishes were purchased from Corning (Corning, NY). AdoMetwas provided from New England Biolabs, prepared in a solution of 5mM H_2SO_4 and 10% ethanol, filtered, and stored at 4°C until use. Lipofectamine 2000, mirVANA PARIS Kit, TaqMan-MiRNA Reverse Transcription Kit, Megaplex RT Primers, TaqMan Universal PCR Master Mix, 384-well TaqMan MiRNA Array CARD, Opti-minimal essential medium (Opti-MEM) LysoTracker Red DND-99 (LTR), ER Tracker Blue DPX, SYBR™ Green PCR Master Mix and Custom DNA Oligos: CHOP, sXBP1, uXBP1, and GAPDH were obtained from Thermo Fisher Scientific (MA).miRNA-888-5p mimics and inhibitors were obtained from Life Technologies (Waltham, MA). Annexin V-FITC Apoptosis Detection kit was purchased from eBioscience (San Diego, CA). Monoclonal antibodies (mAb) to caspase 9, PARP, Bax, Bcl-2, β-actin, α-tubulin and polyclonal antibodies (polyAb) to caspase 6, caspase 7, CHOP, DUSP-1, ERK1/2, phospho-ERK 1/2, LC3B, phospho-P38 and P38 were purchased from Cell Signaling Technology (Danvers, MA).Monoclonal antibodies (mAb) to phospho-JNK and JNK1 were purchased from Santa Cruz Biotechnology (Dallas, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse (GxMu-003-DHRPX) and HRP-conjugated goat anti-rabbit (GtxRb-003-DHRPX) secondary antibodies were obtained from ImmunoReagents. Inc. (Raleigh, NC). All buffers and solutions were prepared with ultrahigh quality water. All reagents were of the purest commercial grade.

Cell cultures and treatments

The laryngeal squamous cancer cell JHU-SCC-011was obtained from the American Type Culture Collection (ATCC, Manassas, VA).JHU-SCC-011 were cultured at 37°C in a 5% CO2 humidified atmosphere and grown in RPMI supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine. Typically, subconfluent cells were seeded at 1.8×10^5/10cmculture dishes. After 24 hours, the cells were

treated with 10% FBS fresh medium containing different concentrations of AdoMet and/or cDDP for 24, 48, and 72 hours. Subsequently, fluctuating cells were recovered from culture medium by centrifugation, whereas adherent cells were collected by trypsinization.

Cell viability assays

JHU-SCC-011 cells were plated in serum-containing media in 96-well plates at the density of $10x10^3$cells/well. After 24 hours incubation, cells were treated with increasing concentration of AdoMet (from 3µM to 1mM) for 24, 48 and 72 hours. Cell viability was assessed by adding MTT solution in PBS to a final concentration of 0.5 mg/mL. Cells were then incubated at 37°C for 4 hour and the MTT-formazan crystals were solubilized in 1 N isopropanol/hydrochloric acid 10% solution at 37°C on a shaking table for 20 min. The absorbance values of the solution in each well were detected at 570 nm using a Bio-Rad IMark microplate reader (Bio-Rad Laboratories, Milan, Italy). All MTT experiments were performed in quadruplicate. Cell viability was expressed as a percentage of absorbance values in treated samples respect to that of control (100%).

Flow cytometry analysis of apoptosis

Annexin V-FITC was used in conjunction with a vital dye PI to distinguish apoptotic (Annexin V-FITC-positive, PI positive) from necrotic (Annexin V-FITC negative, PI positive) cells (177). JHU-SCC-011 were plated in six-multiwell plates at the proper density. After 72 hours, cells, transfected and/or treated, were detached by incubation with EDTA-trypsin, washed twice with PBS, and collected by centrifugation. Then, the cells were resuspended in 200 µl of Binding Buffer 1X and incubated with 5µlAnnexin V-FITC and 10µl PI (20 µg/ml) for 30 min at room temperature, as recommended by the manufacturers. The detection of viable cells, early apoptosis, late apoptosis, and necrotic cells was performed by BD Accuri™ C6 flow cytometer (Becton Dickinson, San Jose, CA). For each sample, 20,000 events were recorded. Analysis was carried out by triplicate determination on at least three separate experiments.

LysoTracker-Red staining

JHU-SCC-011cells were seeded in six-well plates at the proper density and after 24 hours incubation, the cells were were treated with 200 and 300 µM AdoMet for 24 and 48

hours. After treatment, LTR was added to each well for 20 min at 37°C at a final concentration of 0.1 μM in medium. The cells were then washed with PBS and observed by fluorescence microscopy. The fluorescence intensity of the cells was then analyzed by flow cytometry. Then, the cells were detached by incubation with EDTA-trypsin, washed twice with PBS, and collected by centrifugation. The fluorescent emissions were collected as FL1 (linear scale) using BD Accuri™ C6. At least 20,000 events were recorded in log mode. For the quantitative evaluation of LTR, FlowJo software was used to calculate median fluorescence intensities (MFI) by the formula (MFI-treated/MFI-control), where MFI-treated is the fluorescence intensity of cells treated with the various compounds and MFI-control is the fluorescence intensity of untreated cells. Analysis was carried out by triplicate determination on at least three separate experiments.

ER-Tracker Blue-White DPX staining

JHU-SCC-011 cells were seeded in 24-well plates at the density of 6.0×10^3 cells/well, and after 24 hours of incubation, the cells were treated with 200 and 300 μM AdoMet for 48 and 72 hours. To stain ER, the cells were washed in PBS and fixed with 4% paraformaldehyde solution, permeabilized with 0.1% TRITON-X/PBS solution, and incubated with 200 nM ER-Tracker Blue-White DPX in PBS solution for 20 min at 37°C. For positive controls, the cells were exposed for 16 hours to 5μg/ml tunicamycin. Images were collected under a fluorescence microscope (EVOS FL Cell Imaging System, Thermo Scientific, Rockford).

RNA isolation, reverse transcription and qRT-PCR

Total RNA was collected from cultured cells of JHU-SCC-011, treated or not with 300 μM AdoMet, according to manufacturer instructions, by using RNeasy Mini Kit (Qiagen). RNA was treated with DNase (Promega, Milan, Italy) to exclude DNA contamination and 1μg total RNA reverse-transcribed using VILO Super-Script (Invitrogen, Monza, Italy). Gene expression assays were performed on a StepOneThermocycler (Applied Biosystems, Monza, Italy) and the amplifications carried out using SYBR Green PCR Master Mix (Applied Biosystems, Monza, Italy). The reaction conditions were as follows: 95°C for 15 min, followed by 40 cycles of three steps consisting of denaturation at 94°C for 15 s, primer annealing at 60°C for 30 s, and primer extension at 72°C for 30 s. A melting curve analysis was performed from 70°C to 95°C in 0.3°C intervals. Each sample was performed in

triplicate. GAPDH was used to normalize for differences in RNA input. qRT-PCR primer sequences are given in Table 2.

Table 2. Primers sequences for qRT-PCR.

Gene name		**5'-sequence-3'**
CHOP	***Forward***	AGAACCAGGAAACGGAAACAGA
	Reverse	TCTCCTTCATGCGCTGCTTT
uXBP1	***Forward***	CAGCACTCAGACTACGTGCA
	Reverse	ATCCATGGGGAGATGTTCTGG
sXBP1	***Forward***	CTGAGTCCGCAAGCAGGTGCAG
	Reverse	ATCCATGGGGAGATGTTCTGG
GAPDH	***Forward***	GGAGTCAACGGATTTGGTCG
	Reverse	CTTCCCGTTCTCAGCCTTGA

Determination of ROS by DCFH-DA assay

JHU-SCC-011 cells were seeded in six-well plates at the proper density and after 24 hours, the cells were treated with AdoMet 200 and 300 μM for 48and 72 hours, using 100 μM menadione as a positive control for 1 hour at 37°C. After treatment, cells were stained with 10 μM DCFH-DA for 30 min at 37 °C in the dark. Following incubation, cells were washed twice with PBS, trypsinized, resuspended in PBS, and immediately analyzed with a BD Accuri™ C6 flow cytometer (Becton Dickinson, San Jose, CA). For each sample, 20,000 events were recorded. Analysis was carried out by triplicate determination on at least three separate experiments.

Cell transfections

The laryngeal cancer cell lines JHU-SCC-011 were seeded in 6-well plates at the density of 50 x 10^3 cells/well to achieve 80% of confluence. After 24 hours, cells were transfected with 200 nM miR-888-5p inhibitor, diluted in Opti-MEM free medium, supplemented or not (Control) with 300 μM AdoMet, by using Lipofectamine 2000 according to manufacturer's protocol. Lipofectamine was also used alone as a negative control. After 72 hours from transfection, cells were harvested and then subjected to the extraction of the total RNA, preparation of cells lysates and flow cytometry analysis.

MiRNA extraction, detectionand validation by qRT-PCR

Total RNA was isolated from cultured cells treated or not with AdoMet 300 μM, by using the mirVANA PARIS Kit, according to manufacturer instructions. Subsequently, using

the TaqMan MiRNA Reverse Transcription Kit and the Megaplex RT Primers, single-stranded cDNAwas synthesized from total RNA samples. The cDNA targets were amplified by DNA polymerase from the TaqMan Universal PCR Master Mix using sequence-specific primers and probes on the 384-well TaqMan miRNA Array CARD. The array was loaded and run on Applied Biosystems Viia7 instrument (Life Techonologies, USA) by using the default thermal cycling conditions.

To validate the results of the Array CARDs, the expression of miRNAs was independently determined by quantitative real-time PCR (qRT-PCR), using TaqMan miRNA Assays that use looped-primer to accurately detect mature miRNAs. qRT-PCR was performed on a ViiA7™ Real-time PCR system (Applied Biosystems, Darmstadt, Germany). To normalize total RNA samples, the small-nuclear-U6 was selected as an appropriate constitutively expressed endogenous control. Relative expression of the transcripts was measured by using ViiA7™Real-Time PCR software (Applied Biosystems, Darmstadt, Germany).

qRT-PCRdata analysis

Relative quantification of RT-PCR data can be calculated by using the comparative Ct method, a convenient way for determining relative changes in gene expression, with Ct representing the threshold cycle. For fold change, the standard deviation (SD) is calculated to determine if the expression between groups is significant. The endogenous control, small-nuclear-U6 (sn-U6), was used to normalize the expression levels of target genes by correcting differences in the amount of cDNA loaded into PCR reactions.

In details, we normalized the Ct values of a miRNA in each sample to that of the respective endogenous control:

$$Ct = Ct^{miRNA} - Ct^{sn\text{-}U6}.$$

The Ct for each miRNA is calculated using this formula:

$$Ct = Ct^{treated\ cells} - Ct^{control\ cells}$$

Fold changes were calculated as $2^{-\Delta\Delta Ct}$

Preparation of cell lysates

JHU-SCC-011 grown at 37°C for 24, 48 and 72 hours after each treatments, were collected by centrifugation, washed twice with ice-cold PBS, and the pellet was lysed using

100 µl of RIPA Buffer. After incubation on ice for 30 min, the samples were centrifuged at 18,000g in an Eppendorf micro-centrifuge for 30min a 4°C, and the supernatant was recovered. The protein concentration was determined by the Bradford method (178) and compared with BSA standard curve.

Western blotting analysis

Equal amounts of cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes by Trans blot turbo (BIO-RAD). The membranes were washed in 10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% Tween 20 (TBST), and blocked with TBST supplemented with 5% nonfat dry milk. Then, membranes were incubated first with different primary antibodies in TBST and 5% nonfat dry milk, washed, and then incubated with HRP-conjugated secondary antibodies. All primary antibodies were used at a dilution of 1:1,000; all secondary antibodies were used at a dilution of 1:5,000. Blots were then developed using enhanced chemiluminescence detection reagents ECL (Cyanagen, Bologna, IT) and exposed to X-ray film. All films were scanned by using Image J software (National Institutes of Health).

Statistical analysis

Experiments were performed at least three times with replicate samples, except where otherwise indicated. Data are expressed as mean ± standard deviation (SD). The means were compared using analysis of variance (ANOVA) plus Bonferroni's t-test (179). A P-value of <0.05 was considered to indicate a statistically significant result.

REFERENCES

1. **Salvatore F., Borek E., Zappia V., Williams-Ashman H.G., Schlenk F.** (1977) In the Biochemistry of Adenosylmethionine. *Columbia University Press,* pp. 1-588.

2. **Usdin P., Borchardt R.T., Creveling C.R.** (1979) Transmethylation. *Elsevier,* pp. 1-631.

3. **Lu S.C**. (2000) S-Adenosylmethionine. *Int. J. Biochem. Cell Biol,* 32: 391-395.

4. **Fontecave M., Atta M., Mulliez E.** (2004) S-Adenosylmethionine: Nothing goes to waste. *Trends in Biochemical Sciences,* 29: 243–249.

5. **Cantoni G.L.** (1953) S-Adenosylmethionine, a new intermediate formed enzimatically from L-metionine and adenosinetriphosphate. *J. Biol. Chem,* 203: 403-416.

6. **Mato J. M., Alvarez L., Ortiz P., PajaresM. A.** (1997) S-Adenosylmethionine synthesis: molecular mechanisms and clinical implications. *Pharmacology and Therapeutics,* 73: 265-280.

7. **Markham G.D., Pajares M.A.** (2009) Structure-function relationships in methionine adenosyltransferases. *Cellular and Molecular Life Sciences,* 66: 636-648.

8. **Pajares M.A., Markham G.D.** (2011) Methionine adenosyltransferase (S-adenosylmethioninesynthetase). *Advances in Enzymology and Related Areas of Molecular Biology,* 78: 449-452.

9. **Kotb M. and Geller A.M.** (1993) Methionine adenosyltransferase: structure and function. *Pharmacol. Ther.*,59: 125-143.

10. **Markham G.D., Hafner E.W., Tabor C.W., Tabor H.** (1980) S-Adenosylmethioninesynthetase from *Escherichia coli. J. Biol. Chem.,* 255: 9082-9092.

11. **Chiang P.K. and Cantoni G.L.** (1977) Activation of methionine for transmethylation. Purification of the S-adenosylmethioninesynthetase of bakers' yeast e its separation into two forms. *J Biol Chem.,* 252 (13): 4506-4513.

12. **Abe T., Okada G., Teraoka H., Tsukada K.** (1982) Immunological distinction of S-adenosylmethioninesynthetase isozymes from rat liver e kidney. *J Biochem.,* 91(3):1081-1084.

13. **Porcelli M., Cacciapuoti G., Cartení-Farina M., Gambacorta A.** (1988) S-Adenosylmethioninesynthetase in the thermophilic archaebacterium *Sulfolobussolfataricus*. Purification and characterization of two isoforms. *Eur. J. Biochem.*, 177: 273-280.

14. **Porcelli M., Ilisso C.P., De Leo E., Cacciapuoti G.** (2015)Biochemical characterization of a thermostable adenosylmethioninesynthetase from the archaeon

Pyrococcusfuriosus with high catalytic power. *Appl. Biochem. Biotechnol.*, 175(6):2916-33.

15. **Horikawa S., Ozasa H., Ota K., Tsukada K.** (1993) Immunohistochemical analysis of rat methionine adenosyltransferase isozymes in developmental liver. *FEBS Lett.*, 330:307-311.

16. **Yang H.P., Iglesias Ara A., Magilnick N., Xia M., Ramai K., Chen H., Lee T.D., Mato J.M., Lu S.C.** (2008) Expression pattern, regulation and function of methionine adenosyltranferase 2beta alternative splicing variants in hepatoma cells. *Gastroenterology,* 134: 281-291.

17. **Lu S.C., Mato J.M.** (2012) S-Adenosylmethionine in liver health, injury, and cancer. *Physiol. Rev.*, 92(4): 1515-1542.

18. **Mato M.J., Corrales F.J., Lu S.C., Avila M.A.** (2002) S-Adenosylmethionine: a control switch that regulates liver function. *FASEB J.*, 16(1): 15-26.

19. **Vázquez-Chantada M., Fernández-Ramos D., Embade N., Martínez-Lopez N., Varela-Rey M., Woodhoo A., Luka Z., Wagner C., Anglim P.P., Finnell R.H., Caballería J., Laird-Offringa I.A., Gorospe M., Lu S.C., Mato J.M., Martínez-Chantar M.L.** (2010) HuR/methyl-HuR and AUF1 regulate the MAT expressed during liver proliferation, differentiation, and carcinogenesis. *Gastroenterology,* 138(5): 1943-1953.

20. **Hao X., Zhou M., Li H., Angres I.A.** (2017) Novel immunoassays to detect methionine adenosyltransferase activity and quantify S-adenosylmethionine. *FEBS Lett.,* 591(8): 1114-1125.

21. **Maldonado L.Y.,Arsene D.,Mato J.M.,Lu S.C**. (2018) Methionine adenosyltransferases in cancers: Mechanisms of dysregulation and implications for therapy. *ExpBiol Med (Maywood).*, 243(2):107-117.

22. **Frey P.A., Hegeman A.D., Ruzicka F.J.** (2008) The radical SAM superfamily. *Crit. Rev. Biochem. Mol. Biol.,* 43:63-88.

23. **Lu, S.C.** (2009) Regulation of glutathione synthesis. *Mol Aspects Med.*, 30(1-2): 42-59.

24. **Chiang P.K., Gordon R.K., Tal J., Zeng G.C., Doctor B.P., Pardhasaradhi K., McCann P.P.** (1996) S-Adenosylmethionine and methylation. *FASEB J.,* 10(4): 471-480.

25. **Borchardt R.T., Creveling C.R., Ueland P.M.** (1986) Biological Methylation and Drug Design. *Humana Press-Clifton.*, pp.1-457.

26. **Adler J.** (1975) Chemotaxis in *Bacteria*. *Annu. Rev. Biochem.*, 44: 341-56.

27. **Murphy J.1., Mahony J., Ainsworth S., Nauta A., Van Sinderen D.** (2013) Bacteriophage orphan DNA methyltransferases: insights from their bacterial origin, function, and occurrence. *Appl. Environ Microbiol.,* 79(24): 7547-55.

28. **Weissbach A., Ward C., Bolden A.** (1989) Eukaryotic DNA methylation and gene expression. *Curr. Top Cell Regul.,*30: 1-21.

29. **Kredich N.M. and Hershfield M.S.** (1980) Pertubations in S-adenosylhomocysteine and S-adenosylmethionine metabolism: effects on transmethylation. *Adv. Enzym. Regul.,*18: 181-191.

30. **Ueland P.M.,** (1982) Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase. *Pharmacol. Rev.,* 34: 223-253.

31. **Prudova A., Bauman Z., Braun A., Vitvitsky V., Lu SC., Banerjee R.** (2006) S-Adenosylmethionine stabilizes cystathionine β-synthase and modulates redox capacity. *Proc. Natl Acad. Sci.,* 103 (17): 6489-6494.

32. **Mato J., Martínez-López N., Lu SC.** (2013) S-Adenosylmethionine metabolism and liver diasease. *Ann Hepatol.,* 12 (2), 183-189.

33. **Bottiglieri, T.** (2002) S-Adenosyl-L-methionine (SAMe): from the bench to the bedside-molecular basis of a pleiotrophic molecule. *American Journal of Clinical Nutrition,* 76: 1151S-1157S.

34. **Soeken K.L., Lee W.L., Bausell R.B., Agelli M., Berman B.M.** (2012) Safety and efficacy of S-adenosylmethionine (SAMe) for osteoarthritis. *Journal Farmacia Practice,* 51: 425-430.

35. **Pfalzer A.C,Choi S.W,Tammen S.A,Park L.K.,Bottiglieri T.,Parnell L.D.,Lamon-Fava S.** (2014) S-adenosylmethionine mediates inhibition of inflammatory response and changes in DNA methylation in human macrophages. *Physiol Genomics.,* 1;46(17):617-23.

36. **Papakostas G.I., Cassiello C. F., Iovieno N.** (2012) Folates and S-adenosylmethionine for major depressive disorder. *Canadian Journal of Psychiatry. Revue Canadienne de Psychiatrie,* 57: 406-413

37. **Anstee Q.M., Day C.P.** (2012) S-Adenosylmethionine (SAMe) therapy in liver disease: a review of current evidence and clinical utility. *Journal of Hepatology,* 57: 1097-1109.

38. **Mato J.M., Martinez-Chantar M.L., Lu S.C**. (2013) S-Adenosylmethionine metabolism and liver disease. *Annals of Hepatology,* 12: 183-189.

39. **Ramani K., Mato J.M., Lu S.C.** (2011) Role of methionine adenosyltransferase genes in hepatocarcinogenesis. *Cancers,* 3: 1480-1497.

40. **Surtees R., Leonard J., Austin S**. (1991) Association of demyelination with deficiency of cerebrospinal-fluid S-adenosylmethionine in born errors of methyl-transfer pathway. *Lancet,* 338: 1550-1554.

41. **Cheng H.,Gomes-Trolin C., Aquilonius S.M., Steinberg A, Löfberg C., Ekblom J., Oreland L.** (1997) Levels of L-methionine S-adenosyltransferase activity in erythrocytes and concentrations of S-adenosylmethionine and S-adenosylhomocysteine in whole blood of patients with Parkinson's disease. *Exp. Neurol.,* 145: 580-585.

42. **Lee G., Thangavel R., Sharma V.M., Litersky J.M., Bhaskar K., Fang S.M., Do L.H., Andreadis A., Van Hoesen G., Ksiezak-Reding H.** (2004) Phosphorylation of tau by fyn: implications for Alzheimer's disease. *J.Neurosci*., 24(9):2304-12.

43. **Shea T.B.,Chan A.** (2008)S-adenosyl methionine: a natural therapeutic agent effective against multiple hallmarks and risk factors associated with Alzheimer's disease.*J Alzheimers Dis.*,13(1):67-70.

44. **Gomes Trolin C., Regland B., Oreland L.** (1995) Decreased methionine adenosyltransferase activity in erythrocytes of patients with dementia disorders. *Eur. Neuropsychopharmacol.,* 5(2): 107-114.

45. **Gomes-Trolin C., Yassin M., Gottfries C. G., Regland B., Grenfeldt B., Hallman J., Prince J., Oreland L.** (1998) Erythrocyte and brain methionine adenosyltransferase activities in patients with schizophrenia. *J. Neural Transm.,* 105: 1293-1305.

46. **Li N., Zhang H.H., Wang S.H., Zhu W.M., Ren J.A., Li J.S.** (2002) S-Adenosylmethionine in treatment of cholestasis after total parenteral nutrition: laboratory investigation and clinical application. *Hepatobiliary Pancreat. Dis. Int.,* 1(1): 96-100.

47. **Mato J.M., Camara J., Fernandez de Paz J., Caballeria L., Coll S., Caballero A.** (1999) S-Adenosylmethionine in alcoholic liver cirrhosis: a randomized, placebocontrolled, double-blind, multicenter clinical trial. *J. Hepatol.,* 30(6): 1081-1089.

48. **Lu S.C., Mato J.M.** (2008) S-Adenosylmethionine in cell growth, apoptosis and liver cancer. *J. Gastroenterol. Hepatol.*,1: S73-77.

49. **Bian K., Zhang F., Wang T., Zou X., Duan X., Chen G., Zhuge Y.** (2016) S-Adenosylmethionine suppresses the expression of Smad 3/4 in activated human hepatic stellate cells via Rac1 promoter methylation. *Mol. Med. Rep.,* 13(5): 3867-73.

50. **Ansorena E., García-Trevijano E.R., Martínez-Chantar M.L., Huang Z.Z., Chen L., Mato J.M., Iraburu M., Lu S.C., Avila M.A.** (2002) S-Adenosylmethionine and methylthioadenosine are anti-apoptotic in cultured rat hepatocytes but pro-apoptotic in human hepatoma cells. *Hepatology,* 35: 274-280.

51. **Vázquez-Chantada M., ArizU., Varela-Rey M., Embade N., Martínez-Lopez N., Fernández-Ramos D., Gómez-Santos L., Lamas S., Lu S.C., Martínez-Chantar**

M.L., Mato J.M. (2009) Evidence for an LKB1/AMPK/eNOS cascade regulated by HGF, S-adenosylmethionine and NO in hepatocyte proliferation. *Hepatology,* 49: 608-617.

52. **Martínez-López N., Varela-Rey M., Ariz U., EmbadeN., Vazquez-Chantada M., Fernandez-Ramos D., Gomez-Santos L., Lu S.C., Mato J.M., Martinez-Chantar M.L.** (2008) S-Adenosylmethionine and proliferation: new pathways, new targets. *Biochem. Soc. Trans.,* 36: 848-852.

53. **Martínez-Chantar M.L., Vázquez-Chantada M., Garnacho M., Varela-Rey M., Dotor J., Santamaría M., Martínez-Cruz L.A., Parada L.A., Lu S.C., Mato J.M.** (2006) S-Adenosylmethionine regulates cytoplasmic HuR via AMP-activated kinase. *Gastroenterology,* 131: 223-232.

54. **Tomasi M.L., Ramani K., Lopitz-Otsoa F., Rodríguez M.S., Li T.W., Ko K., Yang H., Bardag-Gorce F., Iglesias-Ara A.,FeoF.,Pascale M.R., MatoJ.M.,Lu S.C**. (2010) S-Adenosylmethionine regulates dual-specificity mitogen-activated protein kinase phosphatase expression in mouse and human hepatocytes. *Hepatology,* 51: 2152-2161.

55. **Tomasi M.L., Iglesias-Ara A., Yang H., Ramani K., Feo F., Pascale M.R., Martínez-Chantar M.L., Mato J.M., Lu S.C**. (2009) S-Adenosylmethionine regulates apurinic/apyrimidinic endonuclease 1 stability: implication in hepatocarcinogenesis. *Gastroenterology,* 136: 1025-1036.

56. **Pascale R.M., Simile M.M., Gaspa L., Daino L., SeddaiuM.A., Pinna G., Carta M., Zolo P., Feo F.** (1993) Alterations of ornithine decarboxylase gene during the progression of rat liver carcinogenesis. *Carcinogenesis,* 14: 1077-1080.

57. **Pascale R.M., Simile M.M., De Miglio M.R., Nufris A., Daino L., Seddaiu M.A., Rao P.M., Rajalakshmi S., Sarma D.S., Feo F.** (1995) Chemoprevention by S-adenosyl-L-methionine of rat liver carcinogenesis initiated by 1,2-dimethylhydrazine and promoted by orotic acid. *Carcinogenesis,* 16: 427-430.

58. **Yang H.P., Sadda M.R., Li M., Zeng Y., Chen L., Bae W., Ou X., Runnegar M.T., Mato J.M., Lu S.C.** (2004) S-Adenosylmethionine and its metabolite induce apoptosis in HepG2 cells: role of protein phosphatase 1 and Bcl-xs. *Hepatology,* 40: 221-231.

59. **Cai J., Mao Z., Hwang J.J., Lu S.C.** (1998) Differential expression of methionineadenosyltransferase genes influences the rate of growth of human hepatocellular carcinoma cells. *Cancer Res.,* 58: 1444-1450.

60. **Torres L., Avila M.A., Carretero M.V., Latasa M.U., Caballería J., López-Rodas G., Boukaba A., Lu S.C., Franco L., Mato J.M.** (2000) Liver-specific methionine adenosyltransferase*MAT1A* gene expression is associated with a specific pattern of promoter methylation and histone acetylation: implications for MAT1A silencing during transformation. *FASEB J.,* 14: 95-102.

61. **Frau M., Feo F., Pascale R.M.** (2013) Pleiotropic effects of methionine adenosyltransferase deregulation as determinants of liver cancer progression and prognosis. *J. Hepatol.,*, 59 (4): 830-841.

62. **Li T.W., Peng H., Yang H., Kurniawidjaja S., Panthaki,P., Zheng Y., Mato J.M., Lu S.C.** (2015) S-Adenosylmethionine and methylthioadenosine inhibit β-catenin signaling by multiple mechanisms in liver and colon cancer. *Mol. Pharmacol.*, 87(1): 77-86.

63. **Li T.W., Zhang Q., Oh P., Xia M., Chen H., Bemanian S., et al**. (2009) S-Adenosylmethionine and methylthioadenosine inhibit cellular FLICE inhibitory protein expression and induce apoptosis in colon cancer cells. *Mol. Pharmacol*., 76: 192-200.

64. **Li T.W., Yang H., Peng H., Xia M., Mato J.M., Lu S.C.** (2012) Effects of S-adenosylmethionine and methylthioadenosine on inflammation-induced colon cancer in mice. *Carcinogenesis*, 33: 427-435.

65. **Zhao Y., Li J.S., Guo M.Z., Feng B.S., Zhang J.P**. (2010) Inhibitory effect of S-adenosylmethionine on the growth of human gastric cancer cells *in vivo* and *in vitro*. *Chin. J. Cancer.*, 29: 752-760.

66. **Luo J., Li Y.N., Wang F., Zhang W.M., Geng X.** (2010) S-Adenosylmethionine inhibits the growth of cancer cells by reversing thehypomethylation status of c-MYC and H-RAS in human gastric cancer and colon cancer. *Int. J. Biol. Sci*., 6: 784-795.

67. **Parashar S., Cheishvili D., Arakelian A., Hussain Z., Tanvir I., Khan H.A.** (2015) S-Adenosylmethionine blocks osteosarcoma cells proliferation and invasion in vitro and tumor metastasis in vivo: therapeutic and diagnostic clinical applications. *Cancer Med.*, 4: 732-744.

68. **Ilisso C.P., Sapio L., Delle Cave D., Illiano M., Spina A., Cacciapuoti G., et al.**(2016) S-Adenosylmethionine affects ERK1/2 and Stat3 pathways and induces apotosis in osteosarcoma cells.*J. Cell. Physiol*., 231: 428-435.

69. **Ilisso C.P., Castellano M., Zappavigna S., Lombardi A., Vitale G., Dicitore A., et al**. (2015) The methyl donor S-adenosylmethionine potentiates doxorubicin effects on apoptosis of hormone-dependent breast cancer cell lines. *Endocrine*, 50: 212-222.

70. **Delle Cave D., Desiderio V., Mosca L., Ilisso C.P., Mele L., Caraglia M., et al.**(2018) S-Adenosylmethionine-mediated apoptosis is potentiated by autophagy inhibition induced by chloroquine in human breast cancer cells. *J. Cell. Physiol.*, 233: 1370-1383.

71. **Delle Cave D., Ilisso C.P., Mosca L., Pagano M., Martino E., Porcelli M., Cacciapuoti G.** (2017) The Anticancer Effects of S-Adenosylmethionine on Breast Cancer Cells. *JSM Chemistry*, 2333-6633.

72. **Chik F., Machnes Z., Szyf M.** (2014)Synergistic anti-breast cancer effect of a combined treatment with the methyl donor S-adenosylmethionine and the DNA methylation inhibitor 5-aza-2- deoxycytidine. *Carcinogenesis,* 35:138-144.

73. **Pakneshan P., Szyf M., Farias-Eisner R., Rabbani S.A.**(2004) Reversal of the hypomethylation status of urokinase (uPA) promoter blocks breast cancer growth and metastasis. *J. Biol. Chem.,* 279: 31735-31744.

74. **Shukeir N., Pakneshan P., Chen G., Szyf M., Rabbani S.A.** (2006) Alteration of the methylation status of tumor-promoting genes decreases prostate cancer cell invasiveness and tumorigenesis *in vitro* and *in vivo*.*Cancer. Res.,* 66: 9202-9210.

75. **Yan L., Tingting B., Linxun L., Quangen G., Genhai S., Lei Q.** (2019)S-Adenosylmethionine synergistically enhances the antitumor effect of gemcitabine against pancreatic cancer through JAK2/STAT3 pathway. *Naunyn Schmiedebergs Arch.Pharmacol.,* 392: 615-622.

76. **Sun L., Zhang J., Yang Q., Si Y., Liu Y., Wang Q., Han F., Huang Z.** (2017) Synergistic Effects of SAM and selenium compounds on proliferation, migration and adhesion of HeLa cells. *Anticancer Res.,*37: 4433-4441.

77. **Cattaruzza M.S.,Maisonneuve P.,Boyle P.**(1996) Epidemiology of laryngeal cancer.*Eur. J. Cancer B Oral Oncol*.**,**(5):293-305.

78. **Mastronikolis N.S., Papadas T.A., Goumas, P.D., Triantaphyllidou I.E., Theocharis D.A., Papageorgakopoulou, N., VyniosD.H** (2009) Head and neck: Laryngeal tumors: An overview. Atlas. Genet. Cytogenet. Oncol. Haematol., 13, 888-893.

79. **Conor E., Steuer M.D.,Mark El-Deiry M.D.,Jason R. ,Parks M.D.,Kristin A., Higgins M.D.,Nabil F., Saba M**.(2017)An update on larynx cancer.*C.A. Cancer J.Clin*., 67:31-50.

80. **Cossu A.M.,Mosca L.,Zappavigna S.,Misso G.,Bocchetti M.,De Micco F., Quagliuolo L.,Porcelli M.,Caraglia M.,Boccellino M.**(2019)Long Non-coding RNAs as Important Biomarkers in Laryngeal Cancer and Other Head and Neck Tumours. *Int J Mol. Sci*., 20(14) : 3444.

81. **Cognetti M.D., Weber R.S., Lai S.Y.**(2008). Head and Neck Cancer: An Evolving Treatment Paradigm. *Cancer*, 1911-32.

82. **Dasari S., Tchounwou P.B.** (2014) Cisplatin in cancer therapy: molecular mechanisms of action. *European Journal of Pharmacology,* 5: 364-378.

83. **Baharuddin P.,Satar N.,Fakiruddin K.S.,Zakaria N.,Lim M.N.,Yusoff N.M.,Zakaria Z.,Yahaya B.H.** (2016) Curcumin improves the efficacy of cisplatin by targeting cancer stem-like cells through p21 and cyclin D1-mediated tumour cell inhibition in non-small cell lung cancer cell lines. *Oncol. Rep.*, 35(1):13-25.

84. **Duarte V.M.,Han E.,Veena M.S.,Salvado A.,Suh J.D.,Liang L.J.,Faull K.F.,Srivatsan E.S.,Wang M.B.** (2010) Curcumin enhances the effect of cisplatin in suppression of head and neck squamous cell carcinoma via inhibition of IKKβ protein of the nuclear factor kB pathway. *Mol. Cancer Ther.*,9(10):2665-75.

85. **Liu L., Fan J., Ai G., Liu J., Luo N., Li C., Cheng Z.** (2019) Berberine in combination with cisplatin induces necroptosis and apoptosis in ovarian cancer cells. *Biol Res*.,52(1):37.

86. **Chan M.M., Fong D., Soprano K.J., Holmes W.F., Heverling H.I**. (2003) Inhibition of growth and sensitization to cisplatin-mediated killing of ovarian cancer cells by polyphenolic chemotherapeutic agents. *J Cell Physiol*., 194: 63-70

87. **Bartel D.P.** (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*., 23; 116(2):281-97.

88. **Ambros, V.** (2004) The functions of animal microRNAs. *Nature,* 431: 350-355.

89. **He L., Hannon G.J.** (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev.Genet*, 5: 522-531.

90. **O'Brien J., Hayder H., Zayed Y., Peng C.** (2018) Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation.*Front.Endocrinol.,*3;9:402.

91. **Iorio M.V., Casalini P., Piovan C., Braccioli L., Tagliabue E.** (2011)Breast cancer and microRNAs: therapeutic impact. *Breast.***,**3:S63-70.

92. **Ilisso C.P., Delle Cave D., Mosca L., Pagano M., Coppola A., Mele L., Caraglia M., Cacciapuoti G., Porcelli M.(**2018) S-Adenosylmethionine regulates apoptosis and autophagy in MCF-7 breast cancer cells through the modulation of specific microRNAs.Cancer *Cell Int.,* 4;18:197.

93. **Takamizawa J., Konishi H.,Yanagisawa K.,Tomida S.,Osada H.,Endoh H.,Harano T.,Yatabe Y.,Nagino M.,Nimura Y.,Mitsudomi T.,Takahashi T.** (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival.*Cancer Res.*,1;64(11):3753-6.

94. **Osipov I.D.,Zaporozhchenko I.A.,Bondar A.A.,Zaripov M.M.,Voytsitskiy V.E.,Vlassov V.V.,Laktionov P.P.,Morozkin E.S.** (2016) Cell-Free miRNA-141 and miRNA-205 as Prostate Cancer Biomarkers. *Adv. Exp. Med. Biol.*,924:9-12.

95. **Ju J.** (2010) MiRNAs as biomarkers in colorectal cancer diagnosis and prognosis.*Bioanalysis,* 1; 2(5): 901-906.

96. **Lubov J.,Maschietto M.,Ibrahim I.,Mlynarek A.,Hier M.,Kowalski L.P.,Alaoui-Jamali M.A.,da Silva S.D.**(2017)Meta-analysis of microRNAs expression in head and neck cancer: uncovering association with outcome and mechanisms. *Oncotarget.*15; 8(33): 55511-55524.

97. **Lee R.C., Feinbaum R.L., Ambros V.** (1993) The *C. elegans*heterochronic gene lin-4encodes small RNAs with antisense complementarity to lin-14. *Cell,* 75: 843–854.

98. **Reinhart B.J., Slack F.J., Basson M., Pasquinelli A.E., Bettinger J.C., Rougvie A.E., Horvitz H.R., Ruvkun G.** (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature,* 403: 901-906.

99. **Filipowicz W., Bhattacharyya S.N., Sonenberg N.,** (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature reviews.Genetics,* 9(2), pp. 102-114.

100. **Lagos-Quintana M., Rauhut R., Lendeckel W., Tuschl, T.** (2001).Identification of novel genes coding for small expressed RNAs. *Science,* 294(5543), pp. 853-858.

101. **Lee Y., Kim M., Han J., Yeom K.Y., Lee S., Baek S.H., Kim V.N.** (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J,* 13; 23(20): 4051-4060.

102. **Lee Y., Ahn C., Han J., Choi H., Kim J., Yim J., Lee J., Provost P., Radmark O., Kim S., Kim V.N.** (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature,* 425: 415-419.

103. **Kim V.N.** (2004) MicroRNA precursors in motion: exportin-5 mediates their nuclear export. *Trends. Cell. Biol.,* 14: 156-159.

104. **Hutvagner G., McLachlan J., Pasquinelli A.E., Balint E., Tuschl T., Zamore P.D.** (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science,* 293: 834-838.

105. **Maniataki E., Mourelatos Z.** (2005) A human, ATP-independent, RISC assembly machine fueled by pre-miRNA.*Genes Dev*., 19: 2979-2990.

106. **James S., Parker S., Roe M., Barford D.** (2005) Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. *Nature,*31, 434(7033): 663-666.

107. **Shuibin L., Richard I.G.;** *(*2015) MicroRNA biogenesis pathways in cancer. *Nature Reviews Cancer,* pg 321–333

108. **Felekkis K., Touvana E., Stefanou C., Deltas C.** (2010) MicroRNAs: a newly described class of encoded molecules that play a role in health and disease. *Hippokratia*, 14(4): 236-240.

109. **Broughton J.P., Lovci M.T., Huang J.L., Yeo G.W., Pasquinelli A.E.**(2016)Pairing beyond the Seed Supports MicroRNA Targeting Specificity. *Mol Cell.,*64:320-33.

110. **Zhang J., Zhou W., Liu Y., Liu T., Li C., Wang L.** (2018) Oncogenic role of microRNA-532-5p in human colorectal cancer via targeting of the 5′ UTR of RUNX3.*Oncol Lett*.,15:7215-20.

111. **Dharap A., Pokrzywa C., Murali S., Pandi G., Vemuganti R.** (2013) MicroRNA miR-324-3p induces promoter-mediated expression of RelA gene. *PLoSONE,* 8(11): e79467.

112. **Lu J., Getz G., Miska E.A., Alvarez-Saavedra E., Lamb J., Peck D., Sweet-Cordero, A., Ebert B.L., Mak R.H., Ferrando A.A., Downing J.R., Jacks T., Horvitz H.R. And Golub T.R.** (2005) MicroRNA expression profiles classify human cancers. *Nature,*435(7043), pp. 834-838.

113. **Calin G.A., Sevignani C., Dumitru C.D., Hyslop T., Noch E., Yendamuri S., Shimizu M., Rattan S., Bullrich F., Negrini M., Croce C.M.** (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proceedings of the National Academy of Sciences of the United States of America,* 101(9), pp. 2999-3004.

114. **Kumar M.S., Lu J., Mercer K.L., Golub T.R., Jacks T.,** (2007) Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nature genetics,* 39(5), pp. 673-677.

115. **Lambertz I., Nittner D., Mestdagh P., Denecker G., Vandesompele J., Dyer M.A., Marine J.C.,** (2010). Monoallelic but not biallelic loss of Dicer1 promotes tumorigenesis in vivo. *Cell death and differentiation,* 17(4), pp. 633-641.

116. **Yang, H., Cho, M.E., Li, T.W., Peng, H., Ko, K.S., Mato, J.M., Lu S.C.** (2012) MicroRNAsregulatemethionineadenosyltransferase 1A expression in hepatocellular carcinoma. *J.Clin. Invest.*,123: 285-298.

117. **Koturbash, I., Melnyk, S., James, S.J., Beland, F.A., Pogribny, I.P.** (2013) Role of epigenetic and miR-22 and miR-29b alterations in the downregulation of *MAT1A* and *MTHFR* genes in early preneoplastic livers in rats induced by 2-acetylaminofluorene. *Mol. Carcinog.*,52(4): 318-27.

118. **Xin Y., Zheng L.** (2015) The role of MicroRNAs expression in laryngeal cancer*Oncotarget*.; 15; 6(27): 23297–23305.

119. **Mosca L., Pagano M., Ilisso C.P., Cave D.D., Desiderio V., Mele L., Caraglia M, Cacciapuoti G., Porcelli M.** (2019) AdoMet triggers apoptosis in head and neck squamous cancer by inducing ER stress and potentiates cell sensitivity to cisplatin.*J Cell Physiol.*, 234(8):13277-13291.

120. **Elmore S.** (2007)Apoptosis: a review of programmed cell death. *Toxicol.*,*Patho.*, 35(4): 495-516.

121. **Kerr J.F.R., Wyllie A.H., Currie A.R.** (1972) Apoptosis: A Basic Biological Phenomenon with Wide-ranging Implications in Tissue Kinetics. *Br. J. Cancer,* 26(4): 239-257.

122. **Yigong, S.** (2004) Caspase activation, inhibition, and reactivation: A mechanistic view. *Protein Sci.,* 13(8): 1979-1987.

123. **Dawson, V.L., Dawson, T.M.** (2004) Deadly conversations: Nuclear-mitochondrial cross-talk. *J. Bioenerg.Biomembr.*,36:287-294.

124. **Dawson, S.J., Makretsov, N., Blows, F.M., Driver, K.E., Provenzano, E., Le Quesne, J., Pharoah, P.** (2010) BCL2 in breast cancer: A favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received. *British J. Cancer*, 103(5): 668-675.

125. **Azmi, A.S., Wang, Z., Philip, P.A., Mohammad, R.M., Sarkar, F.H.** (2011) Emerging Bcl-2 inhibitors for the treatment of cancer. *Expert Opinion on Emerging Drugs*, 16(1): 59-70.

126. **Glick, D. Barth**, **S., Macleod**, **K.F**. (2010) Autophagy: cellular and molecular mechanisms. *J. Pathol.*, 221(1): 3-12.

127. **White, E., Di Paola, R.S.** (2009) The double-edged sword of autophagy modulation in cancer. *Clinical Cancer Research*, 15(17): 5308-5316.

128. **Chikte, S., Panchal, N., Warnes, G.** (2014) Use of LysoTracker dyes: A flow cytometric study of autophagy. *Cytometry*, 85(2): 169-178.

129. **Mizushima, N., Yoshimori T.** (2007) How to interpret LC3 immunoblotting. *Autophagy*, 3(6): 542-545.

130. **Tanida, I., Ueno, T., Kominami, E.** (2004) LC3 conjugation system inmammalian autophagy. *Inter. J. Biochem. & Cell Biology*, 36: 2503-2518.

131. **Corazzari M., Gagliardi M., Fimia G.M., Piacentini M.** (2017) Endoplasmic Reticulum Stress, Unfolded Protein Response, and Cancer Cell Fate. *Frontiers in Oncology*, 7:78.

132. **Sano R., Reed J.C.** (2013) ER-stress-induced cell death mechanisms. *Biochim. Biophys. Acta*, 12: 3460-3470.

133. **Yadav R.K., Chae S.W., Kim H.R., Chae H.J.** (2014) Endoplasmic Reticulum Stress and Cancer. *J. Cancer Prev.*, 19(2): 75-88.

134. **Darling N.J., Cook S.J.** (2014) The role of MAPK signalling pathways in the response to endoplasmic reticulum stress. *Biochim. Biophys. Acta*, 1843, 2150-2163.

135. **Shen J.,Zhang Y.,Yu H.,Shen B.,Liang Y.,Jin R.,Liu X.,Shi L.,Cai X.** (2016) Role of DUSP1/MKP1 in tumorigenesis, tumor progression and therapy. *Cancer Med.*5, 2061–2068

136. **Singh I.** (1996) Mammalian peroxisomes: metabolism of oxygen and reactive oxygen species. *Ann NY Acad Sci*., 804:612–627.

137. **Nordberg J., Arner E.S.** (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free RadicBiol Med*., 31(11):1287–1312.

138. **Martin K.R., Barrett J.C.** (2002) Reactive oxygen species as double-edged swords in cellular processes: low-dose cell signaling versus high-dose toxicity. *Hum ExpToxicol.*, 21(2):71–75.

139. **Lewis H., Lance R., Troyer D., Beydoun H., Hadley M., Orians J., Benzine T., Madric K., Semmes O.J., Drake R., Esquela-Kerscher A.** (2014) miR-888 is an expressed prostatic secretions-derived microRNA that promotes prostate cell growth and migration.*Cell Cycle*.,13:227–239. doi: 10.4161/cc.26984.

140. **Huang S., Chen L.** (2014) MiR-888 regulates side population properties and cancer metastasis in breast cancer cells .*BiochemBiophys Res Commun.*,450:1234–1240.

141. **Huang S., Cai M., Zheng Y., Zhou L., Wang Q., Chen L.** (2014) miR-888 in MCF-7 side population sphere cells directly targets E-cadherin. *J Genet Genomics*,41:35–42.

142. **Bader A.G.** (2014) miR-888: Hit it when you see it! *Cell Cycle.*,13:351.

143. **Teng D.H., Perry III W.L., Hogan J.K., Baumgard M., Bell R., Berry S.et al.** (1997) Human mitogen-activated protein kinase 4 as a candidate tumor suppressor. *Cancer Res* 57: 4177–4182.

144. **Su G.H., Hilgers W., Shekher M.C., Tang D.J., Yeo C.J., Hruban R.H. et al.** (1998). Alterations in pancreatic, biliary, and breast carcinomers support MKK4 as a genetically targeted tumor suppressor gene.*Cancer Res*58: 2339–2342.

145. **Chang L., Karin M.**(2001) Mammalian MAP kinase signaling cascades. *Nature;* 410: 37–40.

146. **Davis R.J.** (2000). Signal transduction by the JNK group of MAP kinases.*Cell*103: 239–252.

147. **Bulavin D.V., Fornace Jr A.J.** (2004). p38 MAP kinase's emerging role as a tumor suppressor. *Adv Cancer Res* 92: 95–118.

148. **Lei K., Nimnual A., Zong W.X., Kennedy N.J., Flavell R.A., Thompson C.B.et al.** (2002). The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. Mol Cell Biol 22: 4929–4942.

149. **Maundrell K., Antonsson B., Magnenat E., Camps M., Muda M., Chabert C. et al.** (1997) Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. *J Biol Chem*272: 25238–25242.

150. **Yamamoto K., Ichijo H., Korsmeyer S.J.** (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/*M. Mol Cell Biol*19: 8469–8478.

151. **Fan M., Goodwin M., Vu T., Brantley-Finley C., Gaarde W.A., Chambers T.C.** (2000) Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade.*J Biol Chem*275: 29980–29985.

152. **Deng X., Xiao L., Lang W., Gao F., Ruvolo P., May W.S., Jr.** (2001) Novel role for JNK as a stress-activated Bcl2 kinase. J Biol Chem 276: 23681–23688.

153. **Inoshita S., Takeda K., Hatai T., Terada Y., Sano M., Hata J. et al.**(2002) Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. *J Biol Chem*277: 43730–43734

154. **Zhuang S., Demirs J.T., Kochevar I.E.**(2000) p38 mitogenactivated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J Biol Chem*; 275: 25939-48.

155. **Lee M.W., Park S.C., Yang Y.G., Yim S.O., Chae H.S., Bach J.H., et al.** (2002) The involvement of reactive oxygen species (ROS) and p38 mitogen-activated protein (MAP) kinase in TRAIL/Apo2L-induced apoptosis. *FEBS Lett*; 512: 313-8.

156. **Tan Y., Yu R., Pezzuto J.M. (**2003) Betulinic acid-induced programmed cell death in human melanoma cells involves mitogen-activated protein kinase activation. *Clin Cancer Res;* 9: 2866-75.

157. **Park M.T., Choi J.A., Kim M.J., Um H.D., Bae S., Kang C.M., et al.**(2003) Suppression of extracellular signal-related kinase and activation of p38 MAPK are two critical events leading to caspase-8- and mitochondria-mediated cell death in phytosphingosinetreated human cancer cells. *J Biol Chem*; 278: 50624-34.

158. **Schrantz N., Bourgeade M.F., Mouhamad S., Leca G., Sharma S., Vazquez A.** (2001) p38-mediated regulation of an Fas-associated death domain protein-independent pathway leading to caspase-8 activation during TGFbeta-induced apoptosis in human Burkitt lymphoma B cells BL41. *Mol Biol Cell*; 12: 3139-51.

159. **Deschesnes R.G., Huot J., Valerie K., Landry J.** (2001) Involvement of p38 in apoptosis-associated membrane blebbing and nuclear condensation. *Mol Biol Cell*; 12:1569-82.

160. **Mansouri A, Ridgway LD, Korapati AL, Zhang Q, Tian L, Wang Y, et al.**(2003) Sustained activation of JNK/p38 MAPK pathways in response to cisplatin leads to fas ligand induction and cell death in ovarian carcinoma cells. J Biol Chem.;278(21):19245–56.

161. **Brozovic A., Fritz G., Christmann M., Zisowsky J., Jaehde U., Osmak M., et al.** (2004) Long-term activation of SAPK/JNK, p38 kinase and fas-L expression by cisplatin is attenuated in human carcinoma cells that acquired drug resistance. *Int J Cancer*.;112(6):974–85.

162. **Lafarga V, Cuadrado A, Nebreda AR.** (2007) p18(Hamlet) mediates different p53-dependent responses to DNA-damage inducing agents. *Cell Cycle*.;6(19):2319–22.

163. **Sanchez-Prieto R., Rojas J.M., Taya Y., Gutkind J.S.** (2000) A role for the p38 mitogen-activated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. *Cancer Res*., 60: 2464-2472.

164. **Zhang C., Gao W., Wen S., Wu Y., Fu R., Zhao D., Chen X., Wang B. (**2016) Potential key molecular correlations in laryngealsquamous cell carcinoma revealed by integrated analysis of mRNA, miRNA and lncRNA microarray profiles.*Neoplasma.*63(6):888-900.

165. **Cohen S.M., Lippard S.J.** (2001) Cisplatin: from DNA damage to cancer chemotherapy. *Prog Nucleic Acid Res Mol Biol*.; 67: 93-130.

166. **Kartalou M., Essigmann J.M. (**2001) Mechanisms of resistance to cisplatin. *Mutat Res.*; 478: 23-43.

167. **Rabik C.A., Dolan M.E.** (2007) Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat Rev.*; 33: 9-23

168. **Fu H.Y., Okada K., Liao Y., Tsukamoto O., Isomura T., Asai M.Minamino T.** (2010) Ablation of C/EBP homologous protein attenuates endoplasmic reticulum-mediated apoptosis and cardiac dysfunction induced by pressure overload.*Circulation*122(4):361-9.

169. **Das M., Sabio G., Jiang F., Rincon M., Flavell R.A., Davis R.J.** (2009) Induction of hepatitis by JNK-mediated expression of TNF-α. *Cell,* 136: 249–260

170. **Ventura J.J., Cogswell P., Flavell R.A., Baldwin A.S. Jr, Davis R.J.** (2004) JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. *Genes Dev*18: 2905–2915.

171. **Ozgur R., Uzilday B., Sekmen A. H., TurkanIsmai I.**(2015) The effects of induced production of reactive oxygen species in organelles on endoplasmic reticulum stress and on the unfolded protein response in Arabidopsis. *Ann Bot.*; 116(4): 541–553.

172. **Conklin K.A.** (2004) Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness. *Integr Cancer Ther*.;3:294–300.

173. **Mizutani H., Tada-Oikawa S., Hiraku Y., Kojima M.**, **Kawanishi S.** (2005) Mechanism of apoptosis induced by doxorubicin through the generation of hydrogen peroxide. *Life Sci.;* 76:1439–1453.

174. **Marullo R., Werner E., Degtyareva N., Moore B., Altavilla G., Ramalingam S.S., Doetsch P.W.** (2013) Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. *PLoS One.;* 8:e81162.

175.**Sánchez-Perez, I., Murguía, J.R., Perona, R.** (1998) Cisplatin induces a persistent activation of JNK that is related to cell death. *Oncogene,* 16(4):533-40.

176.**Wang, X., Martindale, J.L., Holbrook, N.J**. (2000) Requirement for ERK activation in cisplatin-induced apoptosis. *J. Biol. Chem*., 275(50):39435-43

177.**Vermes I., Haanen C., Steffens-Nakken H., Reutelingsperger C**. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V.*J. Immunol. Methods*, 184(1):39-51.

178.**Bradford, M.M.** (1976) A rapid e sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.,* 72, 248-254.

*179.***Dunn, O.J.** (1961) Multiple Comparison Among means. *Journal of the American Statistical Association* 56(293), 52-64.