UNIVERSITÀ POLITECNICA DELLE MARCHE

SCUOLA DI DOTTORATO DI RICERCA DELLA FACOLTÀ DI MEDICINA E CHIRURGIA

Curriculum: Scienze biomediche

XV ciclo

OVEREXPRESSION AND SILENCING OF NICOTINAMIDE N-METHYLTRANSFERASE IN HUMAN CANCER CELL LINES

Dottorando:

Dott. Riccardo Seta

Relatore:

Chiar.ma Prof.ssa Monica Emanuelli

Referente:

Chiar.mo Prof. Gian Marco Giuseppetti

Triennio 2013-2016

TABLE OF CONTENTS

1 INTRODUCTION 4

1.1 LUNG CANCER
1.1.1 Epidemiology 5
1.1.2 Risk factors 6
1.1.3 Histopathological classification 8
1.1.4 Staging 10
1.1.5 Clinical manifestations 12
1.1.6 Diagnosis 13
1.1.7 Therapeutic strategies 14

1.2 ORAL CANCER
1.2.1 Epidemiology 17
1.2.2 Risk factors 18
1.2.3 Histopathological classification 19
1.2.4 Staging 20
1.2.5 Clinical manifestations and diagnosis 22
1.2.6 Therapeutic strategies 24

1.3 NICOTINAMIDE N-METHYLTRANSFERASE
1.3.1 Drug metabolism 26
1.3.2 Nicotinamide homeostasis 27
1.3.3 Characterization of human NNMT 28
1.3.4 NNMT polymorphisms 35
1.3.5 NNMT and non-neoplastic diseases 37
1.3.6 NNMT and Parkinson's disease 41
1.3.7 NNMT and neoplastic diseases 43

1.4 AIM OF THE STUDY 51

2 MATERIAL AND METHODS 52

2.1 NNMT SILENCING IN A549 LUNG CANCER CELL LINE
2.1.1 Cell lines and reagents 53
2.1.2 A549 transfection 53

2.2 NNMT OVEREXPRESSION IN HSC-2 ORAL CANCER CELL LINE
2.2.1 Cell lines and reagents 55

2.2.2 Cloning 55
2.2.3 HSC-2 transfection 61

2.3 EFFICIENCY OF NNMT SILENCING AND OVEREXPRESSION
2.3.1 Total RNA extraction and cDNA synthesis 61
2.3.2 Real-Time PCR 62
2.3.3 Western blot analysis 63
2.3.4 Enzyme assay 64
2.3.5 Protein assay 65

2.4 EFFECT OF NNMT SILENCING AND OVEREXPRESSION ON CELL PHENOTYPE
2.4.1 MTT assay 65
2.4.2 Soft agar colony formation assay 66

2.5 EXPRESSION LEVELS OF β-CATENIN, KI-67, AND SURVIVIN ISOFORMS
2.5.1 Qualitative PCR 66
2.5.2 Real-Time PCR 67

2.6 STATISTICAL ANALYSIS 68

3 RESULTS 69

3.1 NNMT SILENCING IN A549 CELLS
3.1.1 Efficiency of NNMT silencing 70
3.1.2 Effect of NNMT silencing on cell proliferation 72
3.1.3 Effect of NNMT silencing on anchorage-independent cell growth 73

3.2 NNMT OVEREXPRESSION IN HSC-2 CELLS
3.2.1 NNMT cloning into pcDNA3 vector 74
3.2.2 Efficiency of NNMT overexpression 76
3.2.3 Effect of NNMT overexpression on cell proliferation 79
3.2.4 Effect of NNMT upregulation on β-catenin, survivin, and Ki-67 80

4 DISCUSSION AND CONCLUSIONS 82

5 LIST OF ABBREVIATIONS 90

6 REFERENCES 94

1. INTRODUCTION

1.1 LUNG CANCER

1.1.1 Epidemiology

Lung cancer is the most common cancer in the world with 1.8 million of new cases in 2012, accounting for 12.9% of the total estimated tumors.

In men, lung cancer is the most common cancer worldwide (1.2 million, 16.7% of the total). It ranks second in more developed regions, whereas predominate in less developed countries with 751,000 cases, constituting 40% of the new cancer cases. The highest incidence rates are in Central and Eastern Europe (53.5 new cases per 100,000 person-years) and Eastern Asia (50.4 new cases per 100,000 person-years), whereas incidence rates are very low in Middle and Western Africa (2.0 and 1.7 new cases per 100,000 person-years, respectively). In women, the incidence rates are generally lower, with a geographical pattern that reflects in part differences in the uptake and consumption of tobacco. The highest lung cancer rates are in Northern America (33.8) and Northern Europe (23.7), but a relatively high incidence has been also estimated among women in Eastern Asia (19.2), despite tobacco smoking is generally rare in these populations. Nevertheless, the high incidence estimated could be related to other known risk factors for lung cancer including air pollution.

Lung cancer is the leading cause of tumor-related death worldwide with 1.59 million deaths (19.4% of the total) reported in 2012. It is the main cause of cancer death among men in both more and less developed regions, whereas in women has surpassed breast cancer as leading cause of cancer mortality in more developed regions [1].

In Western countries, such as United States and the United Kingdom, smoking prevention and cessation programs, as well as advanced medical technologies, have

decreased lung cancer mortality, especially in men. However, in developing countries, where the uptake and consumption of tobacco occurred later, mortality rates are increasing and more efforts to promote smoking cessation and avoid initiation are urgently needed. Nevertheless, at a global level, the mortality patterns of lung cancer closely follow those of incidence, due to the high fatality associated with the disease [2].

The 5-year survival rate is 52.2% for patients with localized disease. Survival declines to 24.3% and 3.6% for patients with regional and distant stage disease, respectively. As the majority of lung cancers (56%) are diagnosed at a distant stage, while only 15% of cases are diagnosed at a local stage, the development of new techniques as well as the identification of reliable biomarkers are required to detect lung cancer at an early stage [3].

1.1.2 Risk factors

Tobacco smoking is the major risk factor for lung cancer, accounting for 80% of the worldwide lung cancer burden in males and at least 50% in females. Tobacco smoke contains many kinds of carcinogenic factors responsible for tumor promoting effects as well as induction of mutations, which cause a 10-fold or greater increased risk of developing lung cancer in cigarette smokers compared to those who have never smoked [4]. Epidemiological studies have shown an approximately linear dose-response relationship between the number of cigarettes smoked per day and lung cancer mortality. A greater than linear increment in incidence or mortality is reported when considering the duration of the smoking habit, probably because of a very high dose of carcinogens that affect a number of steps in carcinogenesis. In former smokers, the risk of developing lung cancer is lower than in continuing smokers, with a correlation

between risk reduction and the length of time the person has quitted smoking. Nevertheless, generally even long-term former smokers have higher risks of lung cancer than those who never smoked [5].

Considering the high correlation between cigarette smoking and lung cancer, most of the worldwide burden of lung cancer could be avoided by promoting smoking cessation and preventing smoking initiation. Stopping tobacco use before middle age avoids more than 90% of the lung cancer risk attributable to tobacco. Smoking cessation can even be beneficial in individuals with an established diagnosis of lung cancer, decreasing side effects from therapy, as well as improving survival and quality of life. Moreover, smoking can alter the metabolism of many chemotherapeutic drugs, highlighting the importance of promoting smoking cessation even after the diagnosis of lung cancer is established.

Although tobacco smoking is considered the most well established risk factor for lung cancer, a number of non-smoking patients have developed this disease, accounting for up to 25% of all lung cancer cases. Deaths from lung cancer are increasing every year in non-smokers, where the disease occupies the seventh place worldwide in terms of mortality.

Environmental tobacco smoke (ETS), or second-hand smoke, is an established cause of lung cancer. The risk from ETS is less than from active smoking, with about a 20-30% increase in lung cancer observed among never smokers married for many years to smokers.

Several other factors are associated with an increased risk of developing lung cancer, including indoor air pollution, genetic susceptibility, and exposure to several occupational and environmental carcinogens, such as asbestos, arsenic, radon,

bis(chloromethyl) ether, hexavalent chromium, mustard gas, nickel, and polycyclic aromatic hydrocarbons.

Occupational observations can provide insights into possible mechanisms of lung cancer induction. The risk of lung cancer among asbestos-exposed workers is increased primarily among those with underlying asbestosis, raising the possibility that the inflammation and scarring produced by this fibrotic lung disease may be the trigger for asbestos-induced lung cancer [4].

The cellular effect of environmental carcinogens can be modulated by polymorphic variations in genes involved in carcinogens' metabolism. Genetic polymorphisms that increase the activity of the cytochrome P450 family enzymes, specifically CYP1A1, or reduce the activity of the glutathione-S transferase family, are associated with the development of lung cancer.

Among first-degree relatives, the risk of lung cancer increases from 2- to 3-fold, suggesting that specific genes and/or genetic variants may contribute to susceptibility to lung cancer. In this regard, increased risk for lung cancer has been reported in individuals with inherited mutations in pRB and p53.

Genome-wide association studies have identified three separate *loci* associated with lung cancer (5p15, 6p21, and 15q25), that include genes involved in acetylcholine nicotinic receptors regulation and telomerase production. Furthermore, the 6q23-25 region was also identified as being linked to lung cancer susceptibility among light and never smokers [6].

1.1.3 Histopathological classification

According to the classification of World Health Organization (WHO), lung cancer is divided into two main histological groups:

- Small-cell lung cancer (SCLC)
- Non-small cell lung cancer (NSCLC)

NSCLC is the most common type of lung cancer and includes squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell carcinoma (LC).

The histological type of NSCLC correlates with the site of origin, reflecting the variation in respiratory tract epithelium of the bronchi to alveoli.

Squamous cell carcinoma accounts for approximately 30% of NSCLC and is the subtype most strongly associated with tobacco smoking, with a higher incidence in men than in women. Squamous cell carcinoma originate in the central part of the lungs, usually near a central bronchus, and may show keratinization and/or intercellular bridges that arise from bronchial epithelium.

Adenocarcinoma is the most common histologic type, accounting for about 40% of lung cancer. Adenocarcinomas exhibit glandular differentiation or mucin production, showing acinar, papillary, bronchioloalveolar, solid features or a mixture of these patterns. Although most cases of adenocarcinoma are associated with smoking, it develops more frequently than any other types of lung cancer in non-smokers, especially in women.

Large cell carcinoma is an undifferentiated non-small cell carcinoma that lacks the cytological and architectural features of small cell carcinoma and glandular or squamous differentiation. Large cell carcinoma accounts for approximately 10% of all lung cancers and predominate in smokers.

Small-cell lung cancer accounts for about 15% of all lung cancer cases, consisting of small cells with scant cytoplasm, ill-defined cell borders, finely granular nuclear chromatin, absent or inconspicuous nucleoli, and a high mitotic count.

SCLC is the most aggressive form of lung cancer, displaying a high incidence rate in men and is commonly associated with smoking [4].

Classification into histopathological subtypes as well as immunohistochemical and molecular characterization of lung cancer provide important information in the prognostic assessment and thus treatment decisions.

At molecular level, a number of gene mutations have been identified in lung cancer. Oncogenes of the Myc family are frequently activated in both SCLC and NSCLC. Mutations that inactivate the tumor suppressor gene TP53, encoding the p53protein, are detected in up to 50% of NSCLC and in over 70% of SCLC. Another common alteration is the inactivation of the suppressor gene RB1, encoding the Rb protein, occurring in over 90% of SCLC cases and 15-30% of NSCLC cases, whereas P16, the other component of the RB pathway, is inactivated in over 50% of NSCLC and in 10% of SCLC. Furthermore, a common genetic event detected in up to 80% in both NSCLC and SCLC is the loss of heterozygosity (LOH) on chromosome 3p. Other mutations are frequent in specific histologic subtypes. KRAS and EGFR mutations are extremely rare in SCLC, whereas are found in 30% and 10% of ADC, respectively. Other driver mutations found in adenocarcinoma include those affecting the serine-threonine kinase BRAF and the lipid kinase PIK3CA, as well as specific chromosomal rearrangements that lead to the activation of tyrosine kinase proteins, such as ALK, ROS1, and RET [4,7].

1.1.4 Staging

Staging of cancer at time of diagnosis is a key factor to provide estimation of prognosis and define treatment strategies. The tumor-node-metastasis (TNM) system is used to classify the NSCLC stage. Such system considers the extent of the primary tumor (T),

the involvement of regional lymph nodes (N), and the presence or absence of distant metastases (M). The most recent revision of the TNM staging system (7th edition) was provided by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) (Table 1). T, N, and M are combined to form different stage groups from I to IV, as shown in Table 2 [8].

Primary Tumor (T)	
T1	Tumor ≤3 cm diameter, surrounded by lung or visceral pleura, without invasion more proximal than lobar bronchus
T1a	Tumor ≤2 cm in diameter
T1b	Tumor >2 cm but ≤ 3 cm in diameter
T2	Tumor >3 cm but ≤7 cm, or tumor with any of the following features:
	Involves main bronchus, ≥2 cm distal to carina
	Invades visceral pleura
	Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung
T2a	Tumor >3 cm but ≤5 cm
T2b	Tumor >5 cm but ≤7 cm
T3	Tumor >7 cm or any of the following:
	Directly invades any of the following: chest wall, diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium, main bronchus <2 cm from carina (without involvement of carina)
	Atelectasis or obstructive pneumonitis of the entire lung
	Separate tumor nodules in the same lobe
T4	Tumor of any size that invades the mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina, or with separate tumor nodules in a different ipsilateral lobe
Regional Lymph Nodes (N)	
N0	No regional lymph node metastases
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Separate tumor nodule(s) in a contralateral lobe; tumor with pleural nodules or malignant pleural or pericardial effusion
M1b	Distant metastasis (in extrathoracic organs)

Table 1. Lung cancer TNM staging.

Stage groupings			
Stage IA	T1a-T1b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T1a,T1b,T2a	N1	M0
	T2b	N0	M0
Stage IIB	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1a,T1b,T2a,T2b	N2	M0
	T3	N1,N2	M0
	T4	N0,N1	M0
Stage IIIB	T4	N2	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1a or M1b

Table 2. Lung cancer stage groups based on T, N, and M.

In patients with SCLC, is generally used the Veteran Administration staging system, a two-stage system that divides SCLC into limited-stage and extensive-stage disease. Limited-stage disease is restricted to the ipsilateral hemitorax and can be encompassed with a single radiation therapy port. The extensive-stage disease includes metastatic disease outside the ipsilateral hemitorax, including malignant pleural effusion, bilateral pulmonary parenchymal, and cardiac tamponade, and cannot be encompassed effectively within a single radiation port. Extensive-stage disease is diagnosed in 60 to 70% of patients with SCLC [9].

1.1.5 Clinical manifestations

Over half of the lung cancer cases are diagnosed at late stage, with locally advanced or metastatic disease. The majority of patients present with signs or symptoms that appear related to primary lesion, metastatic disease, or a paraneoplastic syndrome.

Primary tumors present with symptoms depending on its location. Central or endobronchial growth of primary tumors produce cough, hemoptysis, wheeze, dyspnea,

or postobstructive pneumonitis. Peripheral growth of the primary tumor may cause pain as a result of pleural or chest wall involvement, dyspnea on a restrictive basis, and symptoms of a lung abscess resulting from tumor cavitation.

Regional spread of the tumor may cause superior vena cava obstruction, paralysis of the recurrent laryngeal nerve causing hoarseness, phrenic nerve palsy with paralysis of the diaphragm, pressure on the sympathetic plexus causing Horner's syndrome, and dysphagia resulting from esophageal compression. Other problems of regional spread include pericardial and cardiac effusion, lymphatic obstruction, and lymphangitic spread through the lungs with hypoxemia and dyspnea.

Distant metastatic disease is found in over 50% of patients with squamous cell carcinoma, 80% of patients with adenocarcinoma and large-cell carcinoma, and over 95% of patients with SCLC. Approximately one-third of patients present with symptoms as a result of distant metastases, and such symptoms are correlated with the site of metastatic involvement.

Paraneoplastic syndromes (PNS) occur in 10% of patients with lung cancer and is mainly caused by the production of cytokines or hormones by cancer or as an immune response to cancer. A wide variety of PNS is associated with lung cancer, including hypercalcemia, syndrome of inappropriate antidiuretic hormone secretion and Cushing's syndrome. The symptoms may precede the primary diagnosis by many months, and might be, in some cases, the presenting complaint [4].

1.1.6 Diagnosis

In lung cancer, clinical outcome is related to the stage at diagnosis, thus the identification of high-risk lung cancer individuals at an early disease stage could represent the most effective way of improving survival.

Screening studies conducted in the 1970s based on chest x-rays (CXR) and sputum cytology reported no impact on lung cancer-related mortality in patients characterized as high risk. In contrast to CXR, low-dose computed tomography (LDCT) has emerged as an effective tool for the screening of lung cancer. In 2002, The National Lung Screening Trial (NLST) was initiated to determine whether screening with low-dose computed tomography, as compared with chest radiography, would have reduced mortality from lung cancer among high-risk persons. The NLST demonstrated a 20% reduction in lung cancer-related mortality in the low-dose CT group as compared with the radiography group [10]. Based on these findings, hospital authorities and various organizations recommend lung cancer screening with LDCT in high-risk populations.

Most patients with suspected lung cancer require tissue biopsy to confirm the diagnosis. Tumor tissue may be obtained via bronchial or transbronchial biopsy during fiberoptic bronchoscopy, by needle aspiration or percutaneous biopsy, or lymph node biopsy during mediastinoscopy. Percutaneuos biopsy of a soft tissue mass, pleural or liver lesion, lytic bone lesion, or bone marrow may be obtained in patients with suspected metastatic disease. Because of their central location, squamous cell carcinomas and small-cell carcinomas are readily diagnosed by bronchoscopic biopsy, whereas for peripheral lesions, such as adenocarcinomas and large-cell carcinomas, a transthoracic fine needle aspiration biopsy is generally preferred. Diagnostic accuracy for SCLC versus NSCLC for most specimens is excellent, with lesser accuracy for subtypes of NSCLC [4].

1.1.7 Therapeutic strategies

Treatment of lung cancer may occur in three ways, such as surgery, chemotherapy, or radiotherapy, depending on the stage and the histological type of the disease.

In patients with small-cell lung cancer, the standard treatment is chemotherapy and chest radiotherapy, while surgical resection is not routinely recommended because even limited-stage patients may have occult micrometastases.

Patients with SCLC receive four to six cycles of chemotherapy, with cisplatin or carboplatin plus either etoposide or irinotecan. In limited-stage SCLC patients, thoracic radiation therapy (TRT) is combined with chemotherapy, improving the 3-year survival rate of approximately 5% compared with chemotherapy alone.

With current therapy, patients with limited-stage SCLC have a median survival of 17 months and a 5-year overall survival rate of 12%, while in patients with extended-stage SCLC the median survival ranges from 7 to 11 months, with a 5-year overall survival rate of 2%.

Treatment options of non-small cell lung cancer are based mainly on the stage of cancer. Surgery is the treatment of choice for patients with stage I and II NSCL, who are able to tolerate such procedure. Lobectomy is the standard treatment if the patient can tolerate it, as it offers the best chance for cure, otherwise wedge resection and segmentectomy may be reasonable surgical options. Pneumonectomy is reserved for patients with central tumors and should be performed only in patients with excellent pulmonary reserve. With surgical based therapy, the 5-year survival rates are 60-80% for patients with stage I NSCLC and 40-50% for patients with stage II NSCLC. If patients either refuse or are not suitable candidates for surgery, stereotactic body radiation therapy (SBRT) or another type of radiation therapy may be an option for the treatment of stage I and II NSCLC.

For stage IIIA non-small cell lung cancer, the distinction between non-bulky and bulky mediastinal lymph node (N2) disease is used to select the therapeutic approach. In patients with resectable stage IIIA NSCLC, surgery followed by adjuvant chemotherapy

may be the treatment of choice, while concurrent radiochemotherapy is the standard approach in patients with bulky mediastinal lymph node involvement. Although concurrent radiochemotherapy is associated with improved overall survival compared to sequential treatment, the latter is associated with lower toxicity (including esophagitis and pneumonitis). Therefore, for fit patients, concurrent radiochemotherapy is the preferred treatment approach, whereas sequential treatment may be applied for patients with a poor performance status. For patients who are not candidates for a combined-modality treatment approach, typically due to a poor performance status or a comorbidity that makes chemotherapy untenable, radiotherapy alone may provide a modest survival benefit in addition to symptom palliation [4,7].

1.2 ORAL CANCER

1.2.1 Epidemiology

An estimated 300,400 new cases (2.1% of the total cancer) and 145,400 deaths (1.8% of the total) from oral cavity cancer occurred in 2012 worldwide. The incidence and mortality rate of oral cancer is higher in men than in women. With 199,000 cases estimated in 2012, oral cancer is the 11th most common tumor worldwide among men. There is a wide geographical variation in the incidence of oral cancer, with two-thirds of the total cases occurring in less developed regions. The highest rates are found in Melanesia among both males and females (22.9 and 16.0 new cases per 100,000 person-years, respectively), followed by South-Central Asia, and Central and Eastern Europe, whereas the lowest rates are found in Western Africa and Eastern Asia. The mortality rates follow the geographical pattern of the incidence, with 77% of total deaths estimated in less developed countries [1].

The risk of developing oral cancer increases with age, and the majority of cases occur after the age of 40 years, with a peak at 60 years.

The overall 5-year survival rates for oral cancer has not improved over the past three decades, remaining at approximately 50%. The main reason of this poor prognosis is the diagnostic delay, which leads patients to be treated when the tumor has reached an advanced stage. The survival rate increases up to 80% when the tumor is detected in the early stage [11].

1.2.2 Risk factors

Alcohol consumption and tobacco use are the major risk factors for oral cavity cancer, and when used together, they act synergistically. Smokeless tobacco use (marijuana and occupational exposures) and HPV infection are other etiological factors for oral cancer. Each of these risk factors differently contributes to the burden across countries. Tobacco smoking is estimated to be responsible for about 71% of deaths from oral cancer in high-income regions and 37% in low- and middle-income regions. Incidence rates are increasing among both males and females in several countries of Eastern and Northern Europe as well as among females in Southern and Western Europe, whereas oral cancer rates are decreasing in Asia, Northern America, and Australia among both males and females, reflecting the uptake and consumption of tobacco [2].

Tobacco-specific nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN), and polycyclic aromatic hydrocarbons (PAH) have been causally linked to oral cancer. The metabolism of carcinogens contained in tobacco smoke involves oxygenation and conjugation reactions by P450 enzymes and glutathione-S-transferase, and it has been suggested that genetic polymorphisms in the genes coding for these enzymes may lead to genetic predisposition to tobacco-induced oral cancer [12]. In former smokers the risk of developing oral cancer is consistently lower than in current smokers, with a trend of decreasing risk with increasing number of years since quitting.

Betel quid has been classified as an oral carcinogen by the International Agency for Research on Cancer, with evidence for a dose–response relationship, representing one of the major risk factors for oral cavity cancer in Taiwan, India, and other neighboring countries.

In Western countries, the human papilloma virus (HPV) is associated with a rising incidence of oral cancer, and it is estimated to cause over 50% of oropharyngeal tumors in the United States. HPV is categorized in high-risk and low-risk subtypes, depending on their oncogenic potential. The high-risk HPVs 16 and 18 are the major viral subtypes that have been associated with precancerous and cancerous oral and oropharyngeal epithelial lesions [11].

Several epidemiological studies have shown a relationship between diet and the risk of oral cancer. Low consumption of fruits and vegetables have been correlated with an higher oral cancer rate, whereas more frequent intake of foods containing carotenoids may be protective if included in a balanced diet [12].

1.2.3 Histopathological classification

Oral squamous cell carcinoma (OSCC) is the most common malignancy of oral cavity, accounting for more than 90% of oral cancers, and is divided in well-differentiated, moderately- and poorly-differentiated categories. The assessment of OSCC tumor differentiation is based on the percentage of keratinized tumor cells, with more than 75% keratinization in well-differentiated OSCC, 25-75% keratinization in moderately-differentiated OSCC, and less than 25% in poorly-differentiated OSCC. Poorly differentiated tumors have the worst prognosis, with a 5-year survival rate of 42%, followed by moderately- and poorly-differentiated tumors with a 5-year survival rate of 62% and 74%, respectively. Tumors of the salivary glands represent 2-4% of oral neoplasms, arising from the major (parotid, submandibular, sublingual) or the minor salivary glands (dispersed throughout the upper aerodigestive submucosa). Most parotid gland tumors are benign, whereas slightly more than 50% of submandibular and

sublingual gland tumors, as well as most minor salivary gland tumors are found to be malignant [13].

The World Health Organization defined as potentially malignant disorder (PMD) several lesions and conditions of the oral mucosa with high potential tendency for transformation into malignancy. Conditions described as PMD include erythroplakia, leukoplakia, lichen planus, and oral submucous fibrosis. Erythroplakia and leukoplakia are respectively defined as red and white patch on the oral mucosa, and can be histopathologically classified as hyperplasia, dysplasia, carcinoma in situ, or carcinoma [11].

At molecular level, a variety of gene mutations has been associated with oral cancer. Overexpression of the epidermal growth factor receptor (EGFR) has been reported in oral cancer and has shown to correlate with poor prognosis. P53 tumor suppressor gene mutations are also found frequently, while more than 50% overexpression of p53 has been correlated with a poor prognosis in advanced oral tumors. Frequent alterations associated with oral cancer include the mitotic signaling pathway, Notch pathway, and the PI3K pathway, which is the only mutated cancer gene identified in HPV-positive oral cancer [4].

1.2.4 Staging

Oral cavity and oropharyngeal cancers are classified according to the tumor-node-metastasis (TNM) system of the American Joint Committee on Cancer. T indicates the size of the primary tumors (T1 to T3), whereas T4 usually represent invasion of other tissues of the oral cavity. N describes the size, number, and location on regional lymph node, while M indicates whether tumor has spread to other organs of the body. Distant metastasis are found in less than 10% of patients, mainly involving lung, liver and

bones (Table 3). T, N, and M are combined to form different stage groups from I to IV as shown in Table 4 [14].

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor ≤ 2 cm in greatest dimension
T2	Tumor > 2 cm but ≤ 4 cm in greatest dimension
T3	Tumor > 4 cm in greatest dimension
T4	(lip) Tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face, ie, chin or nose[a]
T4a	Moderately advanced local disease[a] (lip) Tumor invades through the cortical bone, mouth, or skin of the face (ie, chin or nose) (oral cavity) Tumor invades adjacent structures (eg, through cortical bone [mandible or maxilla] into the deep [extrinsic] muscle of the tongue [genioglossus, hyoglossus, palatoglossus, and styloglossus], maxillary sinus, or skin of the face)
T4b	Very advanced local disease Tumor involves masticator space, pterygoid plates, or skull base and/or encases internal carotid artery
Regional lymph nodes (N)	
NX	Regional nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, ≤ 3 cm in greatest dimension
N2	Metastasis in a single ipsilateral lymph node, > 3 cm ≤ 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none > 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none > 6 cm in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, > 3 cm but ≤ 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none > 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none > 6 cm in greatest dimension
N3	Metastasis in a lymph node, > 6 cm in greatest dimension

[a] Superficial erosion alone of bone/tooth socket by gingival primary is not sufficient to classify a tumor as T4.

Distant metastases (M)	
M0	No distant metastasis
M1	Distant metastasis

Table 3. Oral cancer TNM staging

Stage grouping			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

Table 4. Oral cancer stage groups based on T, N, and M.

1.2.5 Clinical manifestations and diagnosis

Clinical manifestations vary depending on the stage and primary site of the tumor. The signs and symptoms of oral cavity carcinomas can be a mouth sore that fails to heal, sudden tooth mobility without apparent cause, or painful lesions. Most early signs are painless and are difficult to detect without a thorough head and neck examination by a dental or medical professional [15].

Tumors of the tongue base or oropharynx can cause decreased tongue mobility and alterations in speech. Cancers of the oropharynx or hypopharynx rarely cause early symptoms, but they may cause sore throat and/or otalgia.

Advanced head and neck cancers in any location can cause severe pain, otalgia, airway obstruction, cranial neuropathies, trismus, odynophagia, dysphagia, decreased tongue mobility, fistulas, skin involvement, and massive cervical lymphadenopathy, which may be unilateral or bilateral [4].

Oral examination involves the inspection of the oral cavity and the palpitation of the floor of the mouth, tongue and neck. Changes in surface texture, loss of surface integrity, color, and size may be suspicious for oral cancer or premalignant lesions. Conventional oral examination has been shown to have high sensitivity, whereas the specificity remains low due to the limited ability to differentiate between benign and precancerous lesions with high risk of progression into invasive oral cancer.

Given the high mortality rate of late stage disease, early detection of oral cancer and premalignant lesions is of paramount clinical importance to improve patient survival and reduce treatment-related morbidity. Detection of lesions may be enhanced by the use of adjunctive aids including vital staining, light-based detection system, histological and cytological techniques, molecular analysis and imaging techniques. Vital tissue staining with toluidine blue is the most used method for early detection of oral cancer and oral potentially malignant disorders. Due to the binding ability to the DNA, toluidine blue staining is used to detect dysplastic and malignant cells, that display higher nucleic acid content than normal, and can be useful to highlight premalignant and malignant oral lesions.

Light-based detection systems work on the assumption that mucosal tissues that have undergone abnormal metabolic or structural changes have different absorbance and reflectance properties when exposed to specific wavelengths. In this light, light-based techniques represent helpful tools to enhance the oral examination process. Tissue biopsy of the suspicious lesion and histopathological examination remain the gold standard for oral cancer diagnosis. An accurate histopathologic diagnosis depends on the clinician doing an appropriate biopsy and providing adequate clinical information, and on the pathologist correctly interpreting the biopsy results. Pathologic evaluation of the presence and degree of epithelial dysplasia is used to assess the malignant risk of

oral premalignant lesions, as low-grade (mild or moderate) and high-grade (severe) dysplasia show a different risk of progression to cancer [16, 17]. Imaging diagnostic techniques provide useful information for screening patients for oral cancer. Computed tomography (CT) scan of the chest and upper abdomen is used to screen for distant metastasis in patients with lymph node involvement. A positron emission tomography (PET) scan may also be helpful to identify or exclude distant metastases. Endoscopy examination, including laryngoscopy, esophagoscopy, and bronchoscopy, is a common diagnostic procedure for oral cancer. Multiple biopsy samples are obtained to establish a primary diagnosis, define the extent of primary disease, and identify any additional premalignant lesions. In patients with lymph node involvement and no visible primary tumor, the diagnosis should be made by lymph node excision [4].

1.2.6 Therapeutic strategies

Treatment of oral cancer depends on the location and the stage of the tumor, as well as the treatment preferences of the individual patient.

Early stage disease is generally treated with single modality therapy by either surgery or radiotherapy (brachytherapy or external beam radiotherapy). Surgery is the traditional approach for small lesions in the oral cavity, while radiotherapy is preferred for unresectable disease, high operative risk patients due to poor performance status, or recurrent disease.

Local or regional advanced diseases are treated with a combined-modality therapy that include surgery followed by adjuvant radiation or chemoradiotherapy.

For unresectable disease, concurrent chemoradiotherapy is the treatment of choice. Meta-analysis of randomized trials has shown that the use of radiation therapy together

with cisplatin improve the overall survival rate in patients with oro- and hypo-pharyngeal carcinomas, as compared to radiotherapy alone.

Patients with recurrent and metastatic disease are generally treated with palliative intent. Chemotherapy using single-agent cisplatin shows a response rate of 30% and a median overall survival of 6-9 month. Combinations of cisplatin/carboplatin with 5-FU or taxane has increased response rates to around 35%, and are considered a standard treatment approach [18, 19].

1.3 NICOTINAMIDE N-METHYLTRANSFERASE

1.3.1 Drug metabolism

Drug metabolism consists of a series of reactions that change the chemical structure of drugs to compounds that can be readily eliminated from the body. During these biotransformation processes, lipid-soluble xenobiotic compounds are enzymatically transformed into polar, water-soluble metabolites which are excreted into urine or bile.

The major site for drug metabolism is the liver, although xenobiotic metabolizing enzyme may be present in other organs such as lung, intestine, and kidney.

Biotransformation of drugs is divided into two phases. Phase I metabolism includes functionalization reactions that introduce polar chemical groups. Phase I reactions involve two groups of enzymes, such as oxidoreductases (cytochrome P450 monooxygenases, flavin monooxygenases, monoamine oxidases, aldehyde dehydrogenase, alcohol dehydrogenase) and hydrolases (amidases, esterases, epoxide hydrolase). In phase II reactions, endogenous compounds or activated xenobiotics metabolites are conjugated with endogenous polar molecules. These conjugation reactions are catalyzed by enzymes, such as glutathione S-transferase, glucuronosyltransferase, sulfotransferase, N-acetyltransferase, and methyltransferase [20].

Among phase II reactions, methyl conjugation is an important pathway in the metabolism of many drugs, xenobiotic compounds, and neurotransmitters.

Methylation of pyridine compounds was first described in 1884 by Wilhelm His. S-adenosyl-L-methionine (SAM) is the methyl donor for this reaction as well as for the methylation of most other drugs and xenobiotics. Human methyltransferases catalyze

the reactions of S-methylation, O-methylation and N-methylation. S-methylation is an important pathway in the biotransformation of drugs such as 6-mercaptopurine, D-penicillamine, and captopril, catalyzed by thiopurine methyltransferase (TPMT, E.C. 2.1.1.67), and thiol methyltransferase (TMT, EC 2.1.1.9). Catechol-O-methyltransferase (COMT, E.C. 2.1.1.6) and phenol O-methyltransferase (POMT, E.C. 2.1.1.25) are involved in the metabolism of several catecholamine neurotransmitters. N-methylation reactions are catalyzed by several N-methyltransferases in human, including histamine N-methyltransferase (HNMT, E.E. 2.1.1.8) and nicotinamide N-methyltransferase (NNMT, E.C. 2.1.1.1) [21, 22].

1.3.2 Nicotinamide homeostasis

The biological effects of vitamin B3, also known as vitamin PP or niacin, are mediated by two functionally related forms of such vitamin: nicotinic acid and nicotinamide. Nicotinamide is a precursor for the synthesis of NAD+ (Nicotinamide Adenine Dinucleotide) and NADP+ (Nicotinamide Adenine Dinucleotide Phosphate), coenzymes that play a key role in ATP production through redox reactions. Both NAD+ and NADP+ are also involved in non-redox mechanisms which lead to the cleavage of the β-N-glycosidic bond to free nicotinamide and include:

- ADP-ribosylation of proteins catalyzed by mono-ADP-ribosyltransferases (ARTs) and poly ADP-ribose polymerases (PARPs) [23].
- calcium mobilization from intracellular stores, mediated by cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) [24].
- deacetylation of histones and transcription factors catalyzed by NAD(+) -dependent histone deacetylases (sirtuins) [25, 26].

Niacin is absorbed in the stomach and intestine by sodium ion-dependent facilitated diffusion at low concentrations, while passive diffusion predominates at higher concentrations [27]. Niacin is found in a variety of foods, but can be also synthesized from tryptophan. In case of low niacin availability, mammals are able to synthesize nicotinamide-containing nucleotides through the kynurenine pathway [28]. The conversion of tryptophan to niacin requires nine steps, whereby 1 mg of nicotinamide was estimated to be produced from approximately 60 mg of dietary tryptophan [29]. Nicotinamide levels may be also regulated through the NAD+ storage in the liver. Hepatic regulation involves the conversion of excess serum nicotinamide to NAD+, whereas hepatic NAD glycohydrolases lead to the release of nicotinamide, which is transported to other tissues. Thus, the liver controls serum levels of nicotinamide, which plays a key role in regulating tissue pyridine nucleotide concentrations [30].

1.3.3 Characterization of human NNMT

Nicotinamide N-methyltransferase (NNMT) is a phase II metabolizing enzyme which plays an important role in the biotransformation and detoxification of many xenobiotic compounds [22]. NNMT catalyzes the N-methylation of nicotinamide, pyridines and other structural analogs, using S-adenosyl-L-methionine as methyl donor, to form positively charged pyridinium ions [31]. The reaction catalyzed by NNMT yields N1-methylnicotinamide (MNA) and S-adenosyl-L-homocysteine (SAH), which is subsequently hydrolyzed to L-homocysteine and adenosine. In human, N1-methylnicotinamide is mostly excreted through urine and partly further converted via catalysis by aldehyde oxidase to N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-5-carboxamide, which are also excreted into urine (Figure 1) [32]. While different N-methyltransferases can catalyze the N-methylation of aza-heterocyclic

compounds, NNMT is the only enzyme known to use nicotinamide as methyl acceptor, playing an important role in nicotinamide catabolism [33, 34].

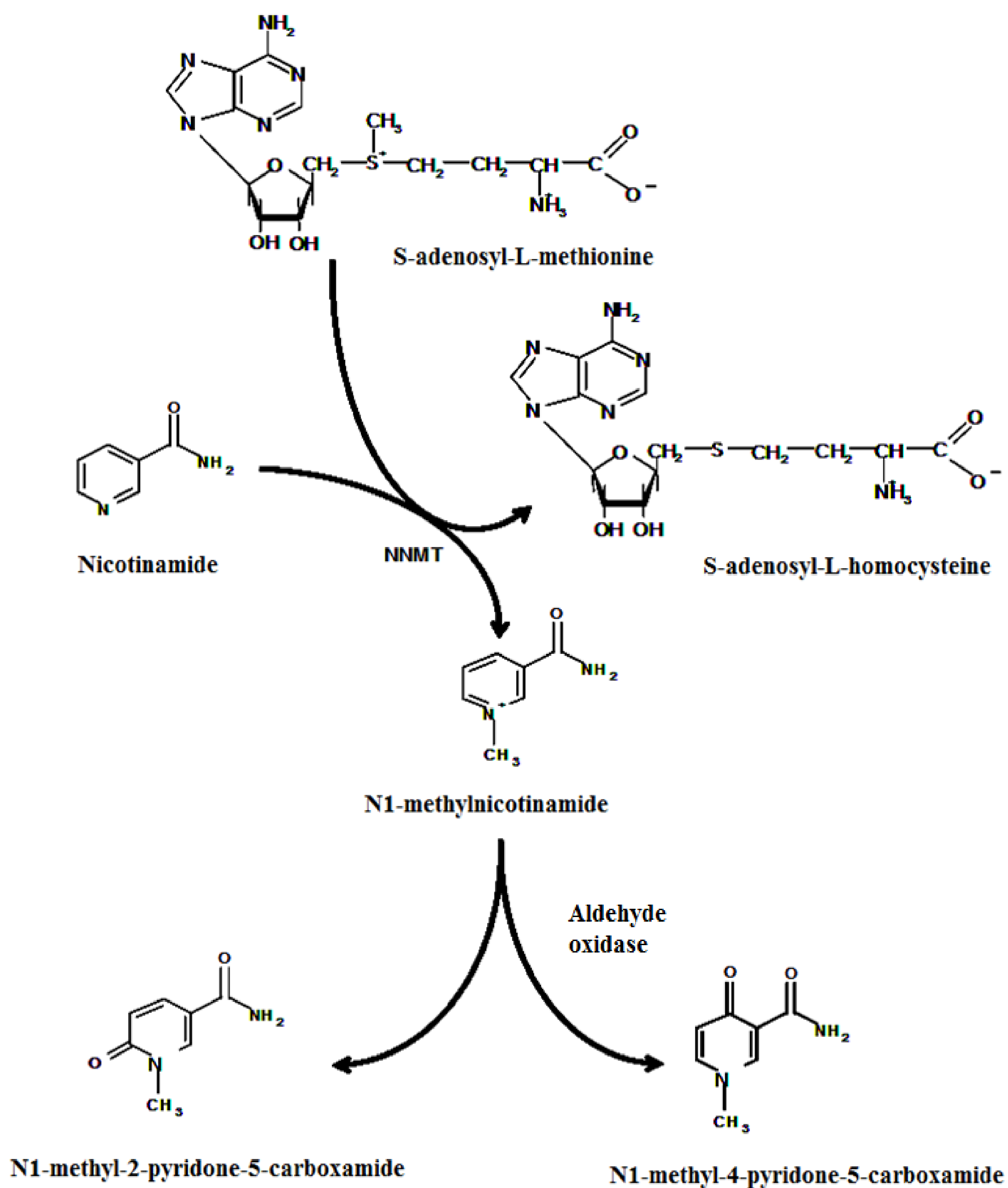

Figure 1. Methylation of nicotinamide and oxidation of N1-methylnicotinamide catalyzed by NNMT and aldehyde oxidase, respectively.

Human NNMT enzyme was first characterized from liver tissue samples, through the development of a radiochemical assay for enzyme activity. The assay was based on the conversion of nicotinamide to radioactively labelled MNA, using [^{14}C-methyl]-S-adenosyl-L-methionine as the methyl donor. The positively charged reaction product was then extracted into 60% isoamyl alcohol in toluene in the presence of the ion-pairing reagent 1-heptanesulfonic acid. The identification of the radioactive product of the enzyme reaction was performed by reversed-phase HPLC, and the radioactivity was measured using a liquid scintillator.

Human hepatic NNMT showed a cytoplasmic distribution with a pH optimum of 7.4. Km values determined for nicotinamide and S-adenosyl-L-methionine were 347 and 1.76 µM, respectively. Human liver NNMT activity was inhibited by both the reaction products, while was not affected by inhibitors of several other methyltransferase. The mean NNMT specific activity, measured in liver biopsy samples, was 51,5 ± 32,5 U/mg, with a large individual variation.

The distribution of activities was bimodal, with approximately 26% of the samples included in a subgroup with high NNMT activity, suggesting that such variations in NNMT activity might be responsible for individual differences in the metabolism and toxicity of pyridine compounds [35]. These observations also raised the possibility that human NNMT activity might be regulated by a genetic polymorphism within the gene sequence [36, 37].

To elucidate such hypothesis, human liver NNMT was partially purified by sequential ion exchange and gel filtration chromatography. The final step in the purification utilized SDS-PAGE, followed by photoaffinity labeling of NNMT that enabled the identification of a protein with a molecular mass of approximately 29 kDa. Partially purified NNMT was then subjected to chemical and enzymatic proteolysis, and the

sequence of the cDNA was obtained from the analysis of the amino acid sequence of generated fragments. The NNMT cDNA possessed a region of 792 bp encoding for a 264 amino acids protein with a calculated molecular mass of 29.6 kDa. The NNMT cDNA was subcloned into the eukaryotic expression vector p91023(B), and the construct obtained was used to transfect COS-1 cells. Substrate kinetic and enzyme inhibition experiments performed on both human liver NNMT and transfected COS-1 cell NNMT, showed a Km values for nicotinamide of 0.43 and 0.38 mM, respectively, and a Km values for S-adenosyl-L-methionine of 1.8 and 2.2 μM, respectively. IC_{50} values for the inhibition of NNMT by N1-methylnicotinamide were 60 and 30 μM for human liver and COS-1 cell-expressed NNMT, respectively [38].

Subsequent experiments were performed to determine the chromosomal localization of the human NNMT gene. The gene is localized on the human chromosome 11q23.1, with a nucleotide sequence of approximately 16.5 kb in length, and consists of 3 exons and 2 introns. Transcription initiation for the NNMT gene occurs at or near a nucleotide located -108 bp upstream from the translation initiation codon, and approximately 30 nucleotides downstream from a TATA box element (Figure 2) [39].

```
1   atggaatcaggcttcacctccaaggacacctatctaagccattttaac  48
1    M   E   S   G   F   T   S   K   D   T   Y   L   S   H   F   N   16
49  cctcgggattacctagaaaatattacaagtttggttctaggcactct  96
17   P   R   D   Y   L   E   K   Y   Y   K   F   G   S   R   H   S   32
97  gcagaaagccagattcttaagcaccttctgaaaatcttttcaagata 144
33   A   E   S   Q   I   L   K   H   L   L   K   N   L   F   K   I   48
145 ttctgcctagacggtgtgaagggagacctgctgattgacatcggctct 192
49   F   C   L   D   G   V   K   G   D   L   L   I   D   I   G   S   64
193 ggccccactatctatcagctcctctctgcttgtgaatcctttaaggag 240
65   G   P   T   I   Y   Q   L   L   S   A   C   E   S   F   K   E   80
241 atcgtcgtcactgactactcagaccagaacctgcaggagctggagaag 288
81   I   V   V   T   D   Y   S   D   Q   N   L   Q   E   L   E   K   96
289 tggctgaagaaagagccagaggcctttgactggtccccagtggtgacc 336
97   W   L   K   K   E   P   E   A   F   D   W   S   P   V   V   T  112
337 tatgtgtgtgatcttgaagggaacagagtcaagggtccagagaaggag 384
113  Y   V   C   D   L   E   G   N   R   V   K   G   P   E   K   E  128
385 gagaagttgagacaggcggtcaagcaggtgctgaagtgtgatgtgact 432
129  E   K   L   R   Q   A   V   K   Q   V   L   K   C   D   V   T  144
433 cagagccagccactgggggccgtccccttaccccggctgactgcgtg 480
145  Q   S   Q   P   L   G   A   V   P   L   P   P   A   D   C   V  160
481 ctcagcacactgtgtctggatgccgcctgcccagacctccccacctac 528
161  L   S   T   L   C   L   D   A   A   C   P   D   L   P   T   Y  176
529 tgcagggcgctcaggaacctcggcagcctactgaagccaggggcttc 576
177  C   R   A   L   R   N   L   G   S   L   L   K   P   G   G   F  192
577 ctggtgatcatggatgcgctcaagagcagctactacatgattggtgag 624
193  L   V   I   M   D   A   L   K   S   S   Y   Y   M   I   G   E  208
625 cagaagttctccagcctcccctgggccgggaggcagtagaggctgct 672
209  Q   K   F   S   S   L   P   L   G   R   E   A   V   E   A   A  224
673 gtgaaagaggctggctacacaatcgaatggtttgaggtgatctcgcaa 720
225  V   K   E   A   G   Y   T   I   E   W   F   E   V   I   S   Q  240
721 agttattcttccaccatggccaacaacgaaggacttttctccctggtg 768
241  S   Y   S   S   T   M   A   N   N   E   G   L   F   S   L   V  256
769 gcgaggaagctgagcagacccctgtga 792
257  A   R   K   L   S   R   P   L   -  264
```

Figure 2. Human NNMT coding sequence. Exons are highlighted with different colours. The amino acid sequence of the encoded protein is shown in single-letter code beneath the nucleotide sequence.

A number of studies reported that the NNMT promoter activity is regulated by transcription factors such as HNF-1β, STAT3, and TGF-β1.

A positive correlation between HNF-1β and NNMT expression levels was found in papillary thyroid cancer cell lines. A putative HNF-1β binding site was identified in the NNMT basal promoter region. Mutations in this site abolished HNF-1β binding and significantly decreased NNMT promoter activity in the HNF-1β-positive papillary thyroid cancer cell line BHP 2-7, suggesting that HNF-1β is able to function as a transcription activator of NNMT promoter [40]. Furthermore, NNMT expression was reduced at both mRNA and protein level in BHP 18-21 papillary thyroid cancer cells treated with depsipeptide, a histone deacetylase inhibitor, and was demonstrated that the inhibition of NNMT by depsipeptide occur at transcription level through the downregulation of the transcription activator HNF-1β [41].

NNMT promoter activity was also correlated to the activation of signal transducer and activator of transcription 3 (STAT3) in colorectal tumor tissues. STAT3 is activated by tyrosine phosphorylation in response to several cytokines including IL-6 and LIF. The NNMT promoter construct was transfected into 293 embryonic kidney and Hep-G2 hepatoma cells, and it was found that the promoter activity was stimulated by LIF and IL-6, respectively. In addition, inhibition of Stat3 in HT29 colon cancer cells with siRNA or curcumin, which inhibited Stat3 phosphorylation, led to a reduction of NNMT expression level [42].

NNMT was also reported to be a target of TGF-β1. Downregulation of TGF-β1 and its target genes, including NNMT, was shown at both mRNA and protein level in human insulinomas compared to normal pancreatic islets [43].

The enzyme is mainly expressed in the liver, while a lower NNMT expression was found in the kidney, lung, placenta, brain, heart, skeletal muscle, and bladder [38].

A recent study revealed the crystal structure of the human NNMT bound to both S-adenosyl-L-homocysteine and nicotinamide. Results obtained by site-directed

mutagenesis confirmed protein features important for the binding of nicotinamide, and the identification of several residues in the active site. Among these, Y20 and D197 residues were shown to be important for nicotinamide recognition and NNMT catalysis (Figure 3) [44].

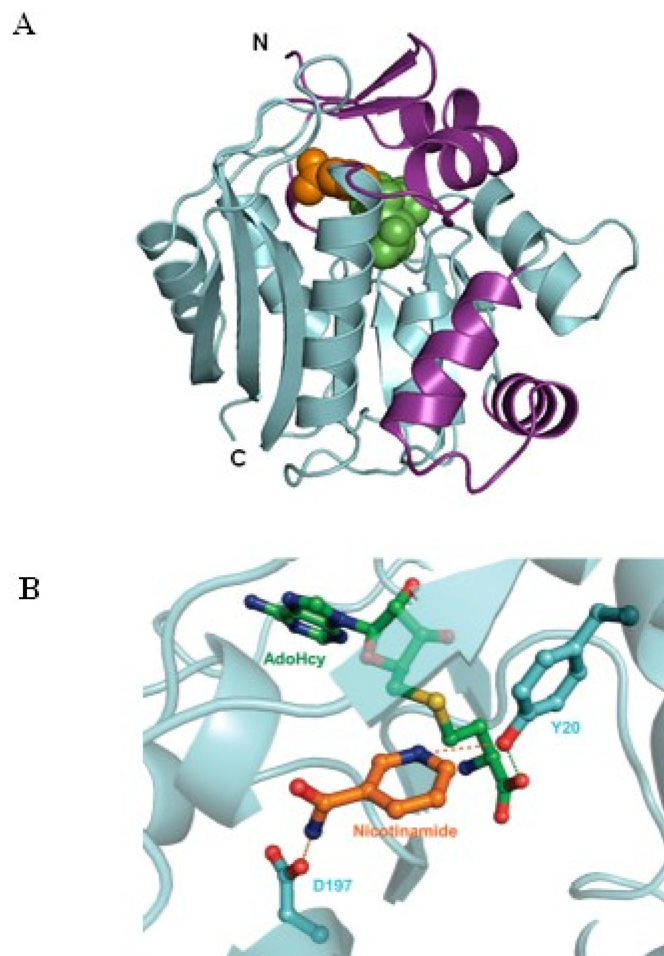

Figure 3. Crystal structure of human NNMT bound to S-adenosyl-L-homocysteine and nicotinamide (A). Y20 and D197 residues in the active site playing a critical role for NNMT catalysis (B).

1.3.4 NNMT polymorphisms

It was hypothesized that the phenotypic differences observed in nicotinamide N-methyltransferase activity may be due to a genetic polymorphism within the coding regions of the gene.

To investigate such hypothesis, the sequence of the three exons, intron 1, and 5'-flanking region of the NNMT gene were tested for the presence of genetic polymorphisms in human liver biopsy samples with low, intermediate or high levels of NNMT activity. No single nucleotide polymorphisms (SNPs) or insertion/deletion events were detected within either the exons or the 5'-flanking region. Eight SNPs were identified within intron 1, but none of these were related to the NNMT activity level. Thus, the individual variability observed in NNMT activity was not attributed to the structure of the enzyme but due to differences in the intracellular NNMT mRNA and protein levels [45, 46].

A genomewide linkage scan for genes affecting variation in plasma Hcy levels was conducted in a study on venous thrombosis, and revealed the presence of a genetic determinant at chromosome 11q23, corresponding to NNMT. Haplotype analyses for 10 single nucleotide polymorphisms (SNPs) in the NNMT gene identified one SNP (rs694539) that was strongly associated with homocysteine levels. Since the SNP is localized in the intron 1, it was hypothesized that this noncoding SNP might be related to the regulation of NNMT transcription [47].

Considering the critical role of NNMT in homocysteine metabolism, a case-control family study, investigated the effect of the NNMT polymorphism on the risk of developing congenital heart defects (CHDs), where maternal hyperhomocysteinemia is a known risk factor. Data obtained showed an eight-fold increased risk for congenital heart defects (CHDs) in children carrying the nicotinamide N-methyltransferase

polymorphism rs694539 (AG/AA genotype) and exposed to periconception medicines and low nicotinamide intake [48].

As maternal hyperhomocysteinemia is considered an important risk factor for neural tube defects, a work conducted in 251 infants with spina bifida and 335 controls, explored the potential correlation between genetic variations of NNMT gene and spina bifida. Although eleven SNPs of the NNMT gene were evaluated, no association between infant NNMT gene variants and risk for spina bifida was found [49].

Several studies suggested an association between abdominal aortic aneurysm (AAA) and hyperhomocysteinemia. Thus, NNMT gene variants may contribute to AAA susceptibility. Using a primer extension based microarray technology, 56 polymorphisms of genes involved in methionine metabolism were evaluated in 423 AAA patients and 423 matched controls. Results obtained showed that seven haplotypes, including one screened for NNMT, were significantly associated with abdominal aortic aneurysm. [50, 51].

A similar study was conducted in 501 young patients who survived ischemic stroke and 1,211 controls. However, no significant correlation between NNMT variants and the pathology was detected [52].

Another work investigated the effects of polymorphisms within folate pathway genes on risk of acute lymphoblastic leukemia (ALL). Results obtained from analyses performed on samples obtained from 245 pediatric ALL patients (cases) and 500 blood bank donors (controls), identified polymorphisms in MTHFR (677 C>T), NNMT (IVS-151 C>T), RFC1 (80G>A) genes. The NNMT IVS-151 TT variant was associated with increased susceptibility to ALL [53].

In a comprehensive association study, the rs694539, previously reported to be correlated with hyperhomocysteinemia, and the rs1941404 NNMT variants were significantly

associated with schizophrenia (SZ). In addition, post-mortem frontal cortex NNMT mRNA levels were 35% lower in schizophrenia patients compared to control subjects, suggesting the involvement of NNMT in the etiology of this disorder [54].

A correlation between the NNMT rs694539 polymorphism and schizophrenia was also found within a Han Chinese female population in a case-control study, confirming the important role of NNMT in the pathogenesis of SZ [55].

Recent studies investigated the potential role of NNMT gene rs694539 variant in the development of diseases correlated with high plasma homocysteine level. The rs694539 NNMT variant was reported to be a genetic risk factor for bipolar disorders, nonalcoholic steatohepatitis, and epilepsy. Furthermore, individuals with the GG genotype displayed protection against nonalcoholic steatohepatitis and epilepsy, whereas the individuals with the AA genotype showed an increased risk for both diseases. Although the biological significance of the rs694539 NNMT variant is unclear, dysregulation of epigenetics, elevated homocysteine levels, as well as dysregulation of the nicotinamide levels may be one of the causes for the development of such pathologies [56, 57, 58].

1.3.5 NNMT and non-neoplastic diseases

Elevated serum levels of N1-methylnicotinamide were detected in patients with cirrhosis, as compared to control subjects, although this disease does not impair the efficiency of nicotinamide methylation [59].

N1-methylnicotinamide has been considered a biologically inactive metabolite of nicotinamide for a long time. However, recent studies have found that MNA possesses anti-inflammatory properties. After topical application of MNA, beneficial therapeutic effects were observed in patients with inflammatory skin diseases, including contact

dermatitis and acne vulgaris [60]. In addition, it was suggested that the anti-inflammatory properties of MNA may be associated with its ability to reduce adherence of pro-inflammatory cells to the vascular endothelium [61].

MNA also displayed anti-thrombotic activity in rats with arterial thrombosis, through a mechanism involving cyclooxygenase-2 (COX-2) and prostacyclin (PGI2). These observations supported the hypothesis that endogenous MNA may regulate thrombosis as well as inflammatory processes characterizing cardiovascular diseases [62].

In a study conducted in mice with atherosclerosis, the progression of the disease was associated with increased hepatic NNMT activity and MNA plasma levels. Therefore, given the anti-thrombotic and anti-inflammatory properties of MNA, high NNMT level may play an important compensatory role in atherosclerosis [63].

Subsequent analysis revealed a significant increase of hepatic NNMT activity and MNA plasma levels during the progression of concanavalinA (ConA)-induced hepatitis in mice, suggesting a potential hepatoprotective effect of MNA through a PGI2-dependent mechanism [64].

NNMT- MNA pathway was shown to be activated during endurance exercise in mice. In addition, data obtained demonstrated that exercise-induced activation of NNMT in the liver involves IL-6, while changes in MNA plasma concentration was partially IL-6-independent. Thus, IL-6 is able to regulate hepatic NNMT activity but also other tissues may determine MNA level in plasma [65].

Changes in NNMT-MNA pathway were also examined in pulmonary arterial hypertension (PAH), a disease associated with inflammatory response. NNMT activity increased progressively in liver and lung in relation to PAH progression, and NNMT response was associated with elevated plasma MNA level. Given the vasoprotective

activity exerted by MNA, the activation of the NNMT-MNA pathway may play a compensatory role in PAH [66].

Upregulation of NNMT gene expression was shown in human renal allograft biopsies with histologic evidence of acute cellular rejection [67]. Furthermore, NNMT expression was detected in endometrial biopsies from patients who became pregnant after intracytoplasmic sperm injection (ICSI) cycles, suggesting a potential involvement of the enzyme in the embryo implantation process [68, 69]. Elevated NNMT expression was demonstrated in endometrial stromal cells in response to factors secreted by macrophages. Therefore, the increased expression of the enzyme observed may be related to the migration and invasion of endometrial cells that occurs during the establishment of endometriosis [70].

In several studies NNMT expression was associated with chronic obstructive pulmonary disease (COPD). High NNMT mRNA expression was observed in quadriceps muscles of patients with COPD [71]. In addition, overexpression of the enzyme in myoblasts significantly increased cell proliferation and migration, as well as reduced protein oxidation and H_2O_2-induced cell death. Therefore, NNMT upregulation observed in the skeletal muscles of patients with COPD may improve myogenesis and defend against oxidative stress [72]. Elevated NNMT levels were also detected in lung tissues of patients with COPD, suggesting a potential association of the enzyme with COPD severity [73].

A recent study highlighted the protective role of NNMT against mitochondrial ROS generation in the pathogenesis of proximal tubular cell (PTC) damage and subsequent renal dysfunction, in patients with refractory proteinuria. In particular, MNA was shown to reduce lipotoxicity-induced oxidative stress and cell damage in kidney proximal tubular cells [74].

In contrast, increased NNMT activity levels were observed in mice treated with phenobarbital, a chemical involved in ROS generation through the induction of cytochrome P450 enzymes. The enhancement of NNMT activity was associated with the depletion of pyridine nucleotides, leading to the attenuation of the NADPH-dependent antioxidant enzymes. Thus, NNMT overexpression may reduce protective systems against oxidative stress [75].

Analysis conducted in murine adipocytes as well as in human and murine adipose tissue samples revealed increased NNMT activity levels and homocysteine release. In this light, high NNMT activity may lead to elevated plasma homocysteine levels, which is considered a risk factor for cardiovascular disease [76].

A recent study reported increased NNMT expression in white adipose tissue (WAT) and liver of obese and diabetic mice. In addition, NNMT knockdown protected mice from diet-induced obesity by augmenting cellular energy expenditure [77]. Subsequent work explored WAT NNMT expression levels in patients with type 2 diabetes and insulin-resistant individuals. Higher WAT NNMT expression was shown in human insulin resistance and type 2 diabetes compared with controls, with a positive correlation observed between NNMT expression and the degree of insulin resistance [78].

Elevated NNMT expression, at both mRNA and serum protein level, was detected in patients with peripheral occlusive arterial disease, as compared to healthy subjects, suggesting a potential use of the enzyme as a biomarker for this pathology [79]. In order to investigate the involvement of the enzyme in metabolic syndrome, NNMT expression was analyzed in the adipose tissue of Wistar Ottawa Karlsburg W (WOKW) rats, which represent an animal model for metabolic syndrome, and Dark Agouti (DA) rats, as control. High NNMT mRNA, protein, and activity levels were observed in adipose

tissue of WOKW rats, supporting the important role of the enzyme in the pathogenesis of this disorder [80].

Although the liver represent the tissue with the strongest NNMT expression, the role played by the enzyme in hepatocytes metabolism is still unclear. A recent study demonstrated that hepatic NNMT levels regulate glucose, lipid and cholesterol metabolism in mice by stabilizing Sirt1 protein, and that the metabolic effects of NNMT are mediated by its product MNA [81].

1.3.6 NNMT and Parkinson's disease

N-methylation is an important pathway in the detoxification of many xenobiotic compounds. However, it has also been reported that this biotransformation increases the toxicity of some substrates. Thus, high levels of NNMT activity in brain may lead to the production of neurotoxic methylpyridinium ions, representing a factor in the etiology of Parkinson disease (PD). To investigate the involvement of NNMT in the occurrence of PD, regional expression of the enzyme were explored in normal human brain tissue. NNMT was found to be expressed solely in neurons and was demonstrated a regional variation in expression levels. High NNMT mRNA levels were shown in the spinal cord, medulla, and temporal lobe, whereas low expression levels were found in the cerebellum, subthalamic and caudate nucleus. Both NNMT protein and enzyme activity were detected in the spinal cord and temporal lobe. Expression of NNMT was compared in PD and non-PD control cerebella and caudate nucleus, resulting significantly elevated in the brains of patients who have died with PD. Furthermore, disease duration was found to be inversely correlated with the level of patient's NNMT expression, suggesting a causative effect in the pathogenesis of the disease [82, 83]. Other studies supported such hypothesis, reporting the neurotoxic effect of N1-methylnicotinamide,

which causes the destruction of complex I subunits of the mitochondrial chain via the induction of free radicals [84, 85, 86, 87].

A recent study was conducted in the human neuroblastoma-derived cell line SH-SY5Y, which lacks of endogenous NNMT, and thus represents an ideal model for investigating the effects of NNMT expression upon cell viability and metabolism. Stable NNMT expression significantly decreased cell death in SH-SY5Y cells, which correlated with increased Complex I activity and ATP synthesis, thus revealing a cytoprotective effect of the enzyme. These effects were replicated by incubation of SH-SY5Y cells with N1-methylnicotinamide, suggesting that this compound is able to mediate the cellular effects of NNMT. Moreover, both NNMT expression and MNA exposure protected SH-SY5Y cells from the toxicity of the Complex I inhibitors MPP+ (1-methyl-4-phenylpyridinium ion) and rotenone. These observations raised the possibility that the NNMT overexpression observed in association with PD patients may represent a stress response of the cell to the underlying pathogenic process [88]. NNMT was also shown to confer protection against mitotoxicity induced by potassium cyanide (KCN), 2,4-dinitrophenol, and 6-hydroxydopamine *in vitro*, and was demonstrated that its neuroprotective effects are not mediated solely via increased N1-methylnicotinamide production, but a variety of mechanisms [89]. Another study conducted in SH-SY5Y and N27 rat mesencephalic dopaminergic cell lines reported the involvement of the enzyme on neuron morphology and differentiation, showing that NNMT expression increased neurite branching [90]. In order to explore the molecular mechanism leading to the observed cytoprotective effects, it was investigated whether sirtuins may mediates the effect of NNMT upon complex I activity. Silencing of sirtuin 3 in NNMT-expressing SH-SY5Y cells demonstrated the important role that sirtuin 3 have in mediating the NNMT-induced complex I activity and ATP synthesis [91]. Given the

high levels of both NNMT and N-methylated β-carbolines (BCs) in PD, in a recent study was investigated whether the enzyme may display N-methyltransferase activity towards BCs. Recombinant NNMT was able to N-methylate the BC norharman (NH) to 2-N-methylnorharman (meNH). Further analyses demonstrated that meNH was less toxic than its precursor NH, and it was shown to increase cell viability and intracellular ATP content in NNMT-expressing SH-SY5Y cells. Taken together, these results suggested the involvement of the enzyme in the detoxification pathway for BCs and that NNMT overexpression may represent a cytoprotective response to the pathogenesis of PD [92].

1.3.7 NNMT and neoplastic diseases

Gene expression profile of glioblastoma multiforme (GBM) tissue samples showed the upregulation of nicotinamide N-methyltransferase in GBMs compared to normal tissues [93]. Moreover, high NNMT activity and N1-methylnicotinamide levels were reported in human glioma cells cultured in the presence of interferon-γ (IFN- γ) [94].

In order to investigate the molecular pathogenesis of thyroid cancer, DNA microarray was used to analyze the gene expression profiles of different human thyroid cancer cell lines, including papillary, follicular, medullary, and anaplastic carcinomas. High NNMT expression was found in papillary cancer cells, whereas weak or no NNMT expression was showed in the other tested cancer cell lines. In addition, NNMT catalytic activity was correlated with the mRNA expression level. Immunohistochemical staining for NNMT of human thyroid specimens revealed strong cytoplasmic reactions in papillary and follicular carcinomas, as weak or scanty reaction was detected in follicular adenomas, colloid goiters, and normal thyroid tissues [95].

A study conducted in a human breast adenocarcinoma cell line revealed the expression of NNMT in adriamycin resistant MCF-7 cells, whereas enzyme level was not detected in parental MCF-7 cells [96, 97]. In a recent study, the cellular effect of NNMT expression was evaluated in different human breast cancer cell lines. NNMT downregulation in Bcap-37 and MDA-MB-231 cells significantly decreased cell growth *in vitro* and tumorigenicity *in vivo*. In addition, NNMT silencing increased ROS production and induced apoptosis via the mitochondria-mediated pathway. The effect of the enzyme on cell proliferation and apoptosis was confirmed by overexpressing NNMT in MCF-7 and SK-BR-3 cell lines, suggesting that NNMT could become an interesting molecular target for breast cancer therapy [98].

Gene expression profile of pancreatic cancer was analyzed in the RNA isolated from pancreatic juice. Increased NNMT expression levels were shown in the pancreatic juice of patients with pancreatic cancer compared with controls [99]. A metabolomics analysis conducted in miR-1291-expressing and control PANC-1 pancreatic cancer cells revealed elevated level of N1-methylnicotinamide in miR-1291-expressing PANC-1 cells. MNA levels were also correlated with NNMT mRNA expression. Stable expression of miR-1291 in PANC-1 cells reduced cell migration, invasion and xenograft tumorigenesis compared to controls. Furthermore, NNMT mRNA level was inversely correlated with pancreatic tumor size in the xenograft mouse model, suggesting the involvement of NNMT in pancreatic tumor progression [100]. Another study explored the biological function of NNMT in pancreatic cancer, showing that NNMT silencing significantly reduced cell proliferation, and suppressed the migration and invasion capacities of PANC-1 cells. In addition, overexpression of NNMT enhanced the survival of PANC-1 cells subjected to either glucose deprivation or glycolytic inhibition, or treated with rapamycin, indicating that NNMT plays an

important role in pancreatic cancer cell metastatic potential and survival under metabolic stress [101]. Elevated NNMT expression levels were found in pancreatic cancer tissue samples compared to paracancerous tissues and chronic pancreatitis. High NNMT expression was correlated with unfavorable clinicopathological characteristics in pancreatic cancer patients and was shown to be an unfavorable prognostic factor of overall survival [102].

Protein expression pattern was explored in healthy and malignant colorectal tissues using a proteomics approach, in order to identify cancer-associated proteins. NNMT was detected at markedly higher levels in colorectal cancer compared with adjacent normal tissue samples. Moreover, elevated levels of NNMT were found in serum from patients with colorectal cancer, suggesting that the enzyme could represent a potential biomarker for the detection of colorectal cancer [103]. A recent study investigated the biological function of NNMT in human colorectal cancer cell lines. Overexpression of NNMT in SW480 cells, which lacks constitutive NNMT expression, increased tumorigenicity *in vitro* and *in vivo*, by inhibiting apoptosis and promoting cell cycle progression. In addition, upregulation of the enzyme increased intracellular ATP level and decreased ROS production. It was shown that the cellular effects of NNMT were mediated by the produced N1-methylnicotinamide, and was suggested a potential involvement of the enzyme in energy balance and ROS production [104]. The effect of the enzyme on 5-fluorouracil (5-FU) sensitivity was also explored in colorectal cancer cells. Overexpression of NNMT in SW480 cells reduced 5-FU-induced apoptosis, and decreased the levels of apoptosis-related proteins, such as caspase-3, caspase-8, and caspase-9. Furthermore, data obtained demonstrated that NNMT inhibited activation of the ASK1-p38 MAPK pathway to decrease apoptosis and enhance 5-FU resistance in colorectal cancer cells. These observations suggested that NNMT plays a role in the

resistance to 5-FU, and may represent a potential therapeutic target for the treatment of colorectal cancer [105].

Comparative proteome analysis performed in gastric cancer samples led to the identification of high NNMT protein levels in stomach adenocarcinoma tissues compared with surrounding normal tissues [106]. In order to identify cancer-associated proteins, subsequent analysis confirmed elevated NNMT level in gastric cancer tissue. Furthermore, the presence of multiple spots of NNMT were found in gastric cancer tissues, while only one spot was detected in both normal and gastric ulcer tissues. These observations suggested that NNMT may receive cancer-specific post-translational modifications in the tumor tissues of the stomach [107]. A recent study examined the prognostic and biological functions of the enzyme in gastric carcinoma. NNMT was found to be upregulated at both mRNA and protein levels in gastric carcinoma tissues compared with normal adjacent tissues, and the gene silencing of the enzyme in MGC803 and BGC823 gastric cell lines was shown to decrease cell proliferation, invasion and migration *in vitro* and *in vivo*. Moreover, NNMT expression was associated with tumor size, lymph node and distant metastasis, TNM stage, and poor overall survival, suggesting the potential role of NNMT as a prognostic factor of gastric carcinoma [108].

In order to explore the involvement of drug-metabolizing enzymes in clear cell renal cell carcinoma (ccRCC), the analysis of gene expression profiles led to the identification of increased NNMT levels in tumor tissue compared with normal renal tissue. In addition, tumor size was inversely correlated with NNMT mRNA levels, suggesting a role of the enzyme in tumor growth [109, 110]. Immunohistochemical analysis confirmed the upregulation of NNMT at protein level in renal cell carcinoma, and high NNMT level was significantly associated with unfavorable prognosis [111].

Elevated NNMT levels was also found in the plasma of RCC patients and in the tumor tissue interstitial fluid compared to normal controls, raising the possibility that NNMT may serve as a diagnostic biomarker for renal cancer [112, 113, 114]. A recent study investigated the potential role of the enzyme in the cellular invasion of ccRCC cells, showing that NNMT gene silencing suppressed invasive capacity *in vitro* and inhibited tumor growth and metastasis *in vivo*. [115].

The expression profile of NNMT was examined in benign and malignant prostate tissues through immunohistochemical analysis, revealing a significant high NNMT level in prostate cancer compared with benign prostate hyperplasia. In addition, clinicopathological analysis showed an inverse correlation between NNMT expression and Gleason score, whereas high NNMT expression was associated with prolonged progression-free survival and overall survival in patients with advanced prostate cancer. These observations suggested a promising role of the enzyme as a prognostic biomarker for prostate cancer [116].

Expression profile analysis of stress-related and DNA repair genes was performed in a radioresistant bladder carcinoma cell line MGH-U1. Data obtained revealed high NNMT mRNA levels compared to its radiosensitive subclone S40b, suggesting a possible role of the enzyme in determining the response to radiotherapy [117]. Subsequent studies were performed to examine the cause of radiation resistance in cancer stem cells (CSCs). Tumorigenic mesenchymal CSC clones overexpressing NNMT displayed elevated radiation resistance, suggesting that high NNMT level could affect the observed radiation response. High NNMT activity may reduce PARP inhibition, exerted by nicotinamide, lowering the cellular level of this compound, thus resulting in DNA damage repair. Furthermore, NAMPT, an enzyme involved in the conversion of nicotinamide into NAD+, was found to be upregulated in the CSC clones

after irradiation. Thus, high NAMPT level may increase NAD+ levels necessary for PARP DNA repair activity [118]. In a recent study, mRNA expression and catalytic activity of NNMT were examined in CSC-enriched populations from Hep-2 cell line. Data obtained showed increased NNMT levels in CSC-enriched populations compared to parental counterpart. Considering the pivotal role of CSCs in tumor initiation and maintenance, these observations suggested an involvement of NNMT in cancer cell metabolism [119].

Gene expression profiles were analized in human bladder cancer cell lines to explore the key molecules involved in cellular invasion. NNMT expression resulted positively correlated with cancer cell migration and tumor stage. Furthermore, NNMT silencing was associated with significant reduction of cell proliferation and migration in bladder cancer cells, indicating a potential role of the enzyme in tumor progression [120]. A recent study revealed higher NNMT expression in bladder urothelial carcinoma (UC) compared with adjacent normal looking tissue, and a marked increase in NNMT enzymatic activity was detected in all cancerous specimens. NNMT expression was also evaluated in exfoliated urinary cells obtained from urine specimens of patients with bladder UC and healthy subjects. Increased NNMT expression at both mRNA and protein level was found in bladder cancer compare to control samples, suggesting a potential application of the enzyme as a biomarker for early and non-invasive detection of bladder cancer [121]. Further analysis showed NNMT upregulation in muscle-invasive bladder cancer compared to non-invasive samples [122].

In order to explore whether NNMT may be a potential biomarker of lung cancer, an ELISA system was set up and revealed a significant increase of serum NNMT level in non-small cell lung cancer (NSCLC) patients compared with healthy donors and patients with chronic obstructive pulmonary disease (COPD). In addition, statistical

analysis showed a higher sensitivity of NSCLC detection when NNMT was used in combination with the carcinoembryonic antigen (CEA), an established lung cancer tumor marker [123]. A recent study evaluated the NNMT expression levels in tumor, tumor-adjacent, and surrounding tissue samples from patients with NSCLC by Real-Time PCR, western blot, catalytic activity assay, and immunohistochemistry. NNMT mRNA and protein levels were significantly higher in tumor compared with both tumor-adjacent and surrounding tissue. Moreover, NSCLC displayed increased activity levels than those determined in both tumor-adjacent and surrounding tissue, suggesting that NNMT may have potential as a molecular marker for NSCLC [124, 125].

NNMT expression and its clinicopathologic relevance were explored in hepatocellular carcinoma (HCC) specimens. NNMT mRNA level was markedly reduced in HCCs compared to non-cancerous surrounding tissues. Moreover, enzyme expression was associated with tumor stage, whereas an inverse correlation was found between NNMT mRNA levels and overall survival, suggesting a potential role of the enzyme as a prognostic factor in hepatocellular carcinoma [126]. Overexpression of NNMT in a human HCC cell line significantly increased cellular invasion and adhesion [127].

The involvement of NNMT was also explored in oral squamous cell carcinoma (OSCC). Increased expression of NNMT was shown in tumor tissues compared with normal mucosa. In addition, NNMT mRNA overexpression in oral cancer was inversely correlated with pT, pathological staging, and lymph node metastasis [128]. Subsequent immunohistochemical analysis revealed a significant inverse correlation between cytosolic NNMT protein levels and the histological grading of the tumor, suggesting that the enzyme may have potential as prognostic marker for OSCC [129]. Recent studies showed higher NNMT activity levels in OSCC than in adjacent normal oral mucosa. Moreover, elevated salivary NNMT expression levels were detected in patients

with OSCC by western blot analysis, thus suggesting that the enzyme could represent a potential biomarker for early diagnosis of oral cancer [130]. In order to explore the role of the enzyme in cancer cell metabolism, the effect of NNMT RNA interference-mediated silencing on cell proliferation was examined in KB and PE/CA-PJ15 cell lines. Downregulation of NNMT expression significantly inhibited cell growth *in vitro* and tumorigenicity *in vivo*, indicating the involvement of the enzyme in tumorigenesis [131, 132].

Elevated NNMT levels were also detected in nasopharyngeal cancer tissues. In addition, NNMT expression was associated with advanced stage and shorter survival, suggesting a potential role of the enzyme as a prognostic factor [133].

A metabolomic analysis showed that high NNMT activity levels affect the methylation potential of cancer cells by consuming methyl units from S-adenosyl-L-methionine. Thus, NNMT-expressing cancer cells display an altered epigenetic state, characterized by hypomethylated histones and cancer-related proteins, as well as increased expression of protumorigenic factors [134, 135].

A recent study reported that NNMT plays an important role in the pluripotency state of human embryonic stem cells (hESCs), regulating the epigenetic landscape during naïve to primed transition [136].

1.4 AIM OF THE STUDY

NNMT overexpression has been reported in a variety of tumors, but the functional significance of alterations in enzyme expression associated with disease remains partly undisclosed.

In this light, the major aim of this project was to explore the role of the enzyme in cancer cell metabolism.

First, to investigate whether the enzyme participates and how it contributes to the tumorigenesis of lung cancer, NNMT expression levels were examined in the human lung cancer cell line A549, by Real-Time PCR, Western blot and catalytic activity assay, and the *in vitro* effect of NNMT knockdown on tumorigenesis was assessed by MTT and soft agar colony formation assays.

Subsequently, to further explore the biological function of the enzyme in cancer cell metabolism, the effect of the induction of NNMT overexpression was evaluated in the human oral cancer cell line HSC-2. Real-Time PCR, Western blot and catalytic activity assay were used to evaluate enzyme overexpression, and the assessment of cell proliferation was performed by MTT colorimetric assay. Subsequent analyses were performed to explore the potential involvement of the enzyme in cellular pathways, such as apoptosis, cell proliferation and cell signaling, evaluating the expression levels of β-catenin, survivin isoforms, and Ki-67.

2. MATERIAL AND METHODS

2.1 NNMT SILENCING IN A549 LUNG CANCER CELL LINE

2.1.1 Cell lines and reagents

The human lung cancer cell line A549, purchased from the America type Culture Collection (ATCC, Rockville, MD, USA), was cultured in DMEM/F12 medium, supplemented with 10% fetal bovine serum and 50 µg/ml of gentamicin, at 37°C in a humidified 5% CO_2 incubator.

2.1.2 A549 transfection

For stable transfection of A549 cells, a set of pLKO.1 vectors containing stem-loop cassettes encoding short hairpin RNA (shRNA) targeted to human NNMT (Sigma-Aldrich, St. Louis, MO) were used. One day before transfection, A549 cells were plated in 500 µl of complete growth medium in 24-well plates ($4x10^4$ cells/well), so that cells were approximately 80% confluent on the time of transfection.

Cells were transfected with three shRNA plasmids against NNMT (pLKO.1-164, 1-330, and 1-711) (Figure 4). Control cells were treated with transfection reagent only (mock). Transfection was performed using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), according to the manufacturer’s instructions.

Briefly, plasmid DNA (0.5 µg per well) was diluted in 25 µl of serum-free medium without antibiotics in a sterile tube. For a 3:1 FuGENE HD Transfection Reagent : DNA ratio, 1.5 µl of the transfection reagent was added to the tube, and the resulting mixture was incubated for 15 minutes at room temperature.

At the end of incubation period, the transfection mixture was added to cells in each well containing 500 µl complete growth medium.

Forty-eight hours after transfection, stably NNMT downregulating clones were selected by maintaining cells in complete medium containing puromycin (1 µg/ml), with medium changes every 48 hours. All subsequent experiments were performed on selected cells growing in complete medium containing puromycin.

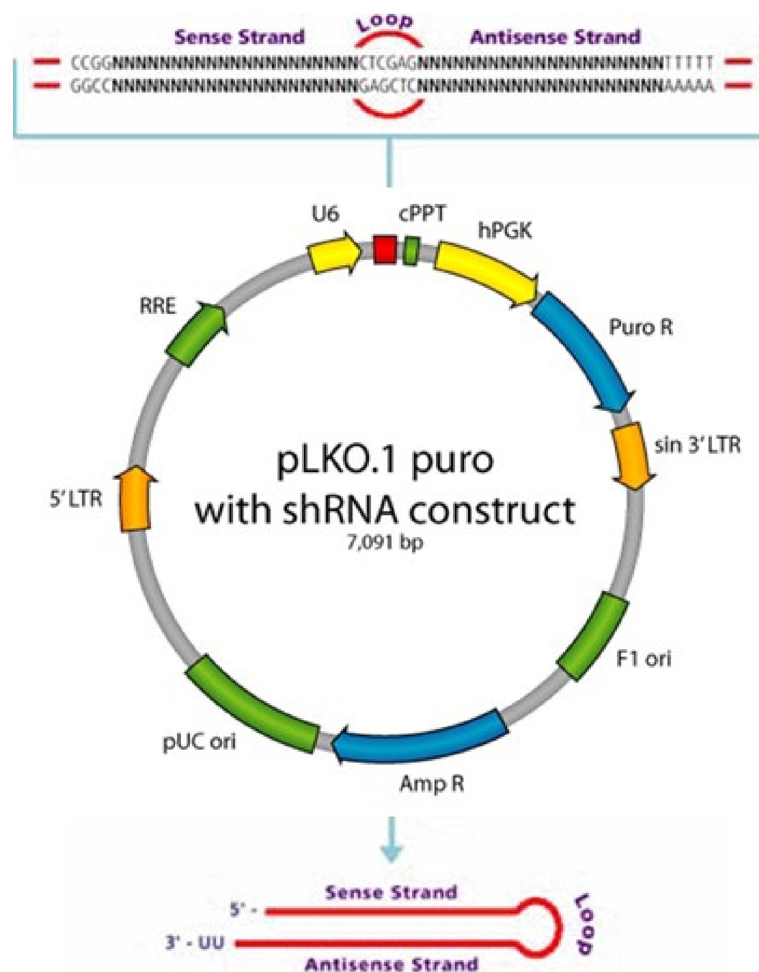

Figure 4. pLKO.1-puro vector. Expression of shRNAs are driven by the human U6 promoter. The vector contains the puromycin resistance (PuroR) gene for selection in mammalian cells.

2.2 NNMT OVEREXPRESSION IN HSC-2 ORAL CANCER CELL LINE

2.2.1 Cell lines and reagents

The human oral cancer cell line HSC-2, purchased from the America type Culture Collection (ATCC, Rockville, MD, USA), was cultured in DMEM/F12 medium, supplemented with 10% fetal bovine serum and 50 µg/ml of gentamicin, at 37°C in a humidified 5% CO_2 incubator.

2.2.2 Cloning

Total RNA was isolated from HSC-2 cells ($1x10^6$) using the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's protocol.
The quantification of total RNA was determined spectrophotometrically by measuring the absorbance at 260 nm. The purity of RNA was assessed by calculating the ratios between absorbance at 260 and 280 nm, as well as absorbance at 260 and 230 nm. The integrity of the purified RNA was confirmed by electrophoresis on denaturing 1% agarose gel.
Total RNA (2 µg) was reverse transcribed in a total volume of 25 µl for 60 minutes at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), using random primers. 1 µl of the reaction mixture was then subjected to PCR with Pfu DNA Polymerase (Biotools, Madrid, Spain) in a total volume of 50 µl, using the primers 5'-TTC GGA TCC ATG GAA TCA GGC TT-3' (NNMT-BamHI-forward) and 5'-TTA CTC GAG TCA CAG GGG TCT GC-3' (NNMT-XhoI-reverse), in order to amplify the human NNMT ORF and to insert BamHI and XhoI restriction sites. PCR was

performed in a thermal cycler using the cycling conditions as follows: initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds (denaturation step), 58°C for 30 seconds (primers annealing step), and 72°C for 1 minute (extension step). The process terminated with a final extension step at 72°C for 5 minutes. The amplified PCR product was subjected to electrophoresis on 1% agarose gel in Tris-acetate-EDTA (TAE) buffer. The DNA PCR product (800 bp) was isolated and purified from the agarose gel, using the NucleoSpin Extract II Kit (Macherey-Nagel, Duren, Germany), according to manufacturer's protocol. The purified DNA fragment was then cloned into the pcDNA3 plasmid vector (Life Technologies, Carlsbad, CA, USA) (Figure 5) to obtain the plasmid construct pcDNA3-NNMT.

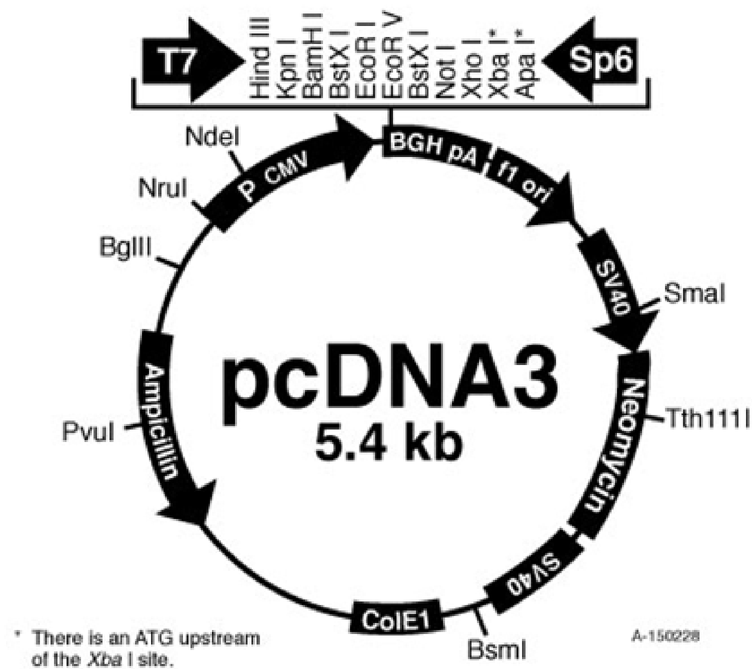

Figure 5. pcDNA3 expression vector. pcDNA3 vector contains the strong CMV enhancer-promoter for high level expression of recombinant proteins in mammalian cells.

1 μg of both insert and vector DNA was digested by using BamHI and XhoI restriction enzymes (Promega), in two separate reaction mixtures. After incubation at 37°C for 1 hour, the digestion products were subjected to electrophoresis on 1% agarose gel in TAE buffer, and the corresponding bands were purified through the NucleoSpin Extract II Kit. The concentrations of the purified products were determined by spectrophotometer, and insert and vector DNA were subsequently subjected to ligation reaction, following a 3:1 insert:vector DNA molar ratio. The amount of insert to use for the ligation reaction was calculated according to the following formula:

$$\text{ng insert} = \frac{\text{ng vector } \times \text{kb of insert}}{\text{kb of vector}} \times \frac{3}{1}$$

Considering the sizes of insert (0.8 kb) and vector (5.4 kb), the amount of the insert DNA to use, for ligation with 100 ng of pcDNA3 vector, resulted 45 ng. Ligation mixture (10 μl) contained 2X Rapid Ligation Buffer, 0.3 Weiss unit of T4 DNA Ligase (Promega) as well as insert and vector DNA, and was incubated for 3 hours at room temperature (Figure 6).

Recombinant plasmid (pcDNA3-NNMT) was used to transformation of E. coli JM109 competent cells, as described below. Briefly, ligation mixture (10 μl) was added to a 200 μl aliquot of bacterial cells and treated as follows:

- incubation for 30 minutes on ice,
- 40-50 seconds at 42°C (heat shock),
- 5 minutes on ice.

After adding 800 μl of sterile LB media, the transformation mixture was incubated at 37°C for 1-2 hours in a shaking incubator. 200 μl of the bacterial culture were then

seeded onto a LB-agar plate containing 0.1 mg/ml ampicillin and incubated overnight at 37°C (Figure 7). The day after, the presence of the recombinant plasmid into JM109 cells was confirmed by subjecting some of the colonies grown on plate to colony-PCR. Reaction mixture (25 µl), prepared according to manufacturer's instructions, contained Green GoTaq Flexi Buffer, $MgCl_{2,}$ dNTP, GoTaq DNA Polymerase (Promega), and primers used for NNMT cloning. PCR was performed in a thermal cycler using the following conditions: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds (denaturation step) and 58°C for 30 seconds (primers annealing and extension step). Amplified products were subjected to electrophoresis on 1% agarose gel in TAE buffer.

Clones harboring recombinant vector were grown in 3 ml of LB media, containing 0.1 mg/ml ampicillin) and incubated overnight at 37 °C in a shaking incubator. The day after, recombinant plasmids were isolated from bacterial pellets using the NucleoSpin Plasmid (Macherey-Nagel, Duren, Germany), and subjected to the analysis of nucleotide sequence.

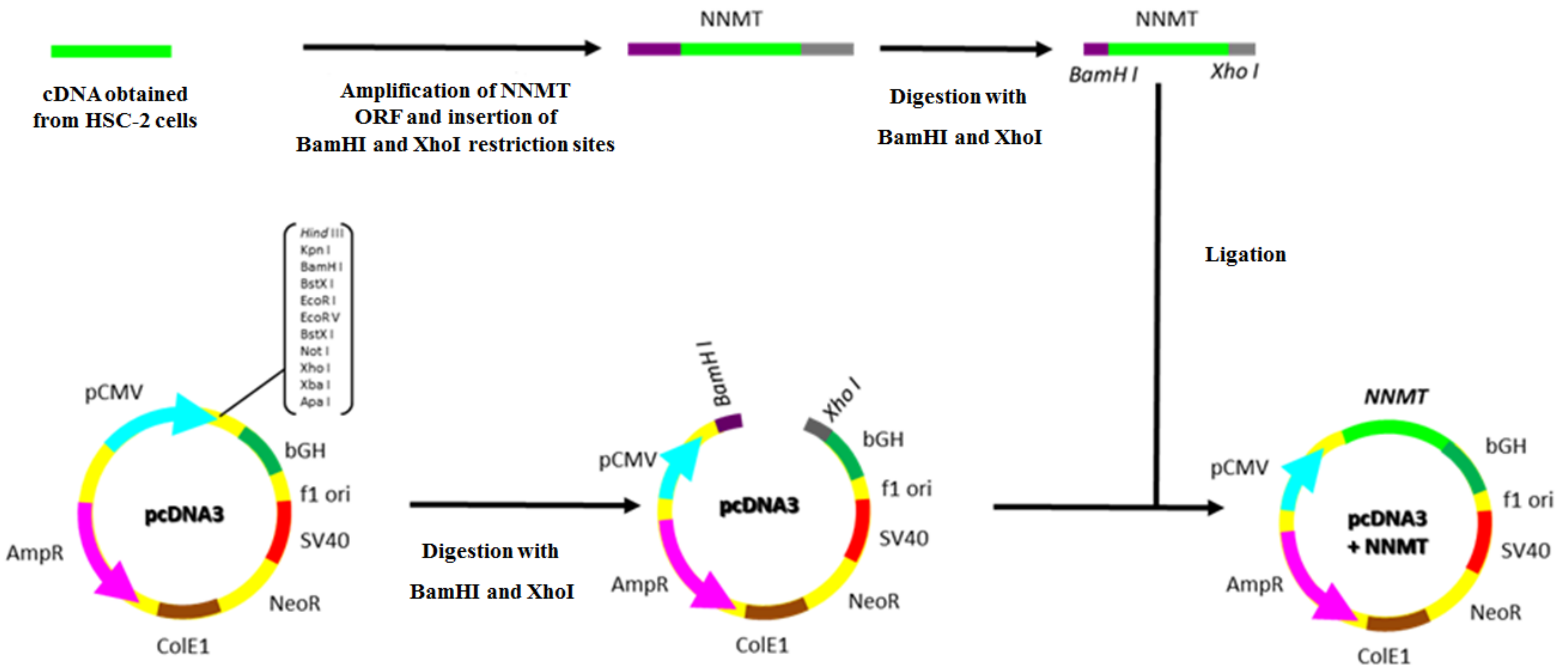

Figure 6. Amplification of the human NNMT ORF, digestion of pcDNA3 vector and insert DNA with BamHI and XhoI, and ligation to obtain the plasmid contruct pcDNA3-NNMT.

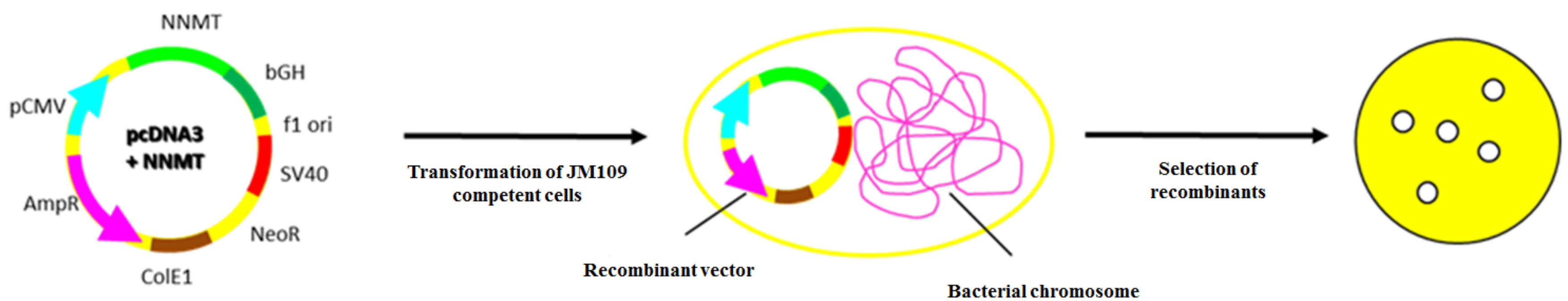

Figure 7. Transformation of E. Coli JM109 competent cells with pcDNA3 recombinant plasmid.

2.2.3 HSC-2 transfection

HSC-2 cells were seeded in 6-well plates (2.4×10^5 cells/well) the day before transfection and were transfected with the pcDNA3-NNMT plasmid vector (3 µg/well). Control cells were transfected with the empty vector (pcDNA3) or treated with transfection reagent only (mock). Transfection was performed using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), as described in the paragraph 2.1.2. Forty-eight hours after transfection, cells were harvested and subjected to further analyses.

2.3 EFFICIENCY OF NNMT SILENCING AND OVEREXPRESSION

2.3.1 Total RNA extraction and cDNA synthesis

Cell pellets (1×10^6 cells) were homogenized in lysis buffer, and total RNA was isolated through the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's protocol.

The quantity and quality of RNA were assessed spectrophotometrically at 260 nm and 280 nm, and confirmed by electrophoresis, as previously described. Total RNA (2 µg) was reverse transcribed in a total volume of 25 µl for 60 minutes at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) using random primers. cDNA samples were used to perform subsequent Real-Time PCR analysis.

2.3.2 Real-Time PCR

To evaluate NNMT mRNA expression quantitatively, a Real-Time PCR assay was performed using a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). cDNA, generated as described above, was used as template. To avoid false-positive results caused by amplification of contaminating genomic DNA in the cDNA preparation, all primers were selected to flank an intron, and PCR efficiency was tested for both primer pairs and found to be close to 1. The primers used were 5′-GAA TCA GGC TTC ACC TCC AA-3′ (forward) and 5′-TCA CAC CGT CTA GGC AGA AT-3′ (reverse) for NNMT, and 5′-TCC TTC CTG GGC ATG GAG T-3′ and 5′-AGC ACT GTG TTG GCG TAC AG-3′ for β-actin.

Both genes were run in duplicate for 40 cycles at 94°C for 30 seconds and 58°C for 30 seconds, using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested in triplicate with the reference gene β-actin for data normalization to correct for variations in RNA quality and quantity. Direct detection of PCR products was monitored by measuring the fluorescence produced by EvaGreen dye binding to double strand DNA after every cycle. These measurements were then plotted against cycle numbers. The parameter threshold cycle (Ct) was defined as the cycle number at which the first detectable increase above the threshold in fluorescence was observed. Following NNMT gene silencing in A549 cells, fold changes in relative gene expression were calculated by $2^{-\Delta(\Delta Ct)}$ [137], where ΔCt = Ct (NNMT)- Ct (β-actin) and Δ(ΔCt) = ΔCt (transfected cells) - ΔCt (mock). Concerning NNMT overexpression in HSC-2 cells, Δ(ΔCt) = ΔCt (pcDNA3 or pcDNA3-NNMT) - ΔCt (mock).

2.3.3 Western blot analysis

Western Blot experiments were performed to evaluate NNMT protein expression level. Cell pellets ($2x10^6$ cells) were suspended in 200 μl lysis buffer (phosphate buffered saline containing 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin) and homogenized by passing 3-5 times through a 30 gauche needle attached to a 1 ml syringe. After centrifugation at 16000 x g for 10 minutes at 4°C, the supernatant containing the protein extract was collected. Samples containing 50 μg protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli method [138], using a running gel of 15% polyacrylamide. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 250 mA for 30 minutes, using a wet transfer method. PVDF membranes were blocked overnight at 4°C in 1X phosphate buffered saline (PBS) solution containing 5% nonfat dry milk, and 0.05% tween-20. After washing three times with 1X PBS containing 0.05% tween-20, the membranes were incubated with rabbit polyclonal antibody against NNMT (Sigma-Aldrich, St. Louis, MO, USA) (1:1000 dilution) for 1 hour, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) (1:2000 dilution) for 1 hour. NNMT protein was visualized using enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal of NNMT protein detected in blots was acquired using ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules, CA, USA).

2.3.4 Enzyme assay

An HPLC-based catalytic assay was performed to analyze NNMT activity. Cell pellets ($5x10^6$ cells) were suspended in 200 µl of cold lysis buffer (50 mM Tris-HCl, pH 8.6, 2 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1% Nonidet P-40) and ½ volume glass beads. The suspension was vigorously vortexed for 2 minutes and then chilled on ice for 2 minutes. The homogenate was centrifuged at 16000 x g for 10 minutes at 4°C. The supernatant was collected and kept on ice until assayed. The standard assay mixture contained 50 mM Tris-HCl, pH 8.6, 1 mM dithiothreitol, 5 mM nicotinamide, 0.5 mM S-adenosyl-L-methionine and the appropriate amount of enzyme sample to reach a final volume of 350 µl. The reaction was started by adding S-adenosyl-L-methionine. Incubations were performed at 37°C for 30 and 60 minutes. The reaction was stopped by adding 100 µl assay mixture to 50 µl ice-cold 1.2M $HClO_4$. After 10 minutes at 0°C, proteins were removed by 1 minute of centrifugation in a microfuge and 130 µl perchloric acid supernatant were then neutralized by adding 35 µl 0.8M K_2CO_3. The $KClO_4$ so formed was removed by centrifugation. 100 µl of the neutralized supernatant was injected into a high performance liquid chromatography system 10 Dvp-UV-Vis photodiode array detector (Shimadzu, Duisburg, Germany) using a 250×4.6 mm inner diameter Supelcosil LC-18-S 5 µm reversed phase column. Elution conditions were as previously described [139]. Enzyme activities were tested by measuring the amount of N1-methylnicotinamide produced, as determined by the peak areas of the separated compound, with 1U activity representing the formation of 1 nmol N1-methylnicotinamide per hour of incubation at 37°C. The lower detection limit for NNMT catalytic activity assay was 0.01 U/mg.

2.3.5 Protein assay

Protein concentration was measured by the Bradford method, using bovine serum albumin as the standard [140].

2.4 EFFECT OF NNMT SILENCING AND OVEREXPRESSION ON CELL PHENOTYPE

2.4.1 MTT assay

Cell proliferation was determined using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzyme activity of the intact mitochondria of living cells. After transfection, A549 and HSC-2 cells were seeded in 96-well plates ($5x10^3$ cells/well). Cells were allowed to attach overnight and cell proliferation was evaluated for up to 4 days by measuring the conversion of the tetrazolium salt MTT to formazan crystals. Briefly, 10 µl of MTT reagent (5 mg/ml in phosphate buffered saline) was added to 200 µl of growth medium and cells were incubated for 4 hours at 37°C. The medium was removed and 200 µl of isopropanol were added. The amount of formazan crystals formed directly correlates with the number of viable cells. The reaction product was quantified by measuring the absorbance at 570 nm using an ELISA plate reader. Experiments were repeated three times. Results were analyzed and expressed as percentage of the control (control equals 100% and corresponds to the absorbance value of each sample at time zero), and

presented as mean values ± standard deviation of three independent experiments performed in triplicate.

2.4.2 Soft agar colony formation assay

Anchorage-independent cell growth was determined on the basis of cell proliferation in soft agar. Soft agar colony-forming assay was performed in triplicate. A549 cells were seeded in 24-well plates ($2x10^4$ cells/well) in DMEM/F12 medium containing 0.35% low melting point (LMP) agar overlying a 0.7% LMP agar layer. The cells were cultured at 37°C in 5% CO_2 for 30 days. Every 7 days 500 µl of fresh medium was added to each well and visible colonies were photographed.

2.5 EXPRESSION LEVELS OF β-CATENIN, KI-67, AND SURVIVIN ISOFORMS

2.5.1 Qualitative PCR

The cDNA obtained as described in the paragraph 2.3.1 was used to analyze the expression levels of β-catenin, Ki-67, and survivin isoforms in HSC-2 cells, by Real-Time PCR. In order to accomplish to this task, primer pairs were designed for the amplification of the above genes, by using cDNA samples from HSC-2 as templates. Preliminary PCR analyses were performed using GoTaq DNA Polymerase (Promega) and the cycling conditions described in the paragraph 2.2.2. The oligonucleotide sequences of primers for survivin isoforms, β-catenin, Ki-67 were reported in Table 5. PCR products were subjected to electrophoresis on 2% agarose gel in TAE buffer.

2.5.2 Real-Time PCR

Real-Time PCR was performed to quantify the expression level of β-catenin, Ki-67, survivin main isoform, survivin ΔEx3 isoform, and survivin 2B isoform, using conditions as described in the paragraph 2.3.2.

Target gene	Sequence
Survivin	forward 5 -ATGACGACCCCATAGAGGAAC-3’ reverse 5’-CCTTTGCAATTTTGTTCTTGGC-3’
Survivin 2B	forward 5’-CACTGAGAACGAGCCAGACT-3’ reverse 5’-ATGTTCCTCTCTCGTGATCC-3’
Survivin ΔEx3	forward 5’-GACCACCGCATCTCTACATTC-3’ reverse 5’-ATTGTTGGTTTCCTTTGCATG-3’
Survivin 2α	forward 5’-GCCCAGTGTTTCTTCTGCTT-3’ reverse 5’-GTTCAAAACAAAGCCCATCG-3’
Survivin 3B	forward 5’-TGGACAGAGAAAGAGCCAAGA-3’ reverse 5’-CTTCCAGTCCCTCCCTGAAT-3’
β-catenin	forward 5’-AACAGGAAGGGATGGAAGGT-3’ reverse 5’-ATACCACCCACTTGGCAGAC-3’
Ki-67	forward 5’-GACATCCGTATCCAGCTTCC-3’ reverse 5’-CCGTACAGGCTCATCAATAAC-3’

Table 5. Primers used for both qualitative PCR and Real-Time PCR.

2.6 STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism software version 6.00 for Windows (GraphPad Prism Software, San Diego, CA, USA). Differences between groups were determined using the Mann-Whitney and Kruskal-Wallis test. A p-value < 0.05 was accepted as statistically significant.

3. RESULTS

3.1 NNMT SILENCING IN A549 CELLS

3.1.1 Efficiency of NNMT silencing

In order to modulate NNMT expression for functional assays, A549 cell line was stably transfected with three shRNA plasmids targeting different regions of NNMT mRNA, and control cells were treated with transfection reagent only (mock), as described in the paragraph 2.1.2. The specific effects of shRNA treatment on NNMT expression were evaluated at both mRNA and protein levels by Real-Time PCR, Western blot and catalytic activity assay.

Real-Time PCR showed a significant ($p < 0.05$) downregulation of NNMT in cells transfected with pLKO.1-164 (3.85-fold reduction), pLKO.1-330 (3.70-fold reduction) and pLKO.1-711 (3.33-fold reduction) plasmids compared with control cells (Figure 8A). Results obtained by quantitative Real-Time PCR were confirmed at protein level by Western blot analysis. Lanes loaded with equal protein amounts displayed markedly decreased NNMT expression in transfected compared to control cells (Figure 8B). An HPLC-based catalytic activity assay was performed to analyze NNMT activity. In keeping with the results of Real-Time PCR and Western blot, the levels of NNMT specific activity, expressed in U/mg protein, were significantly higher in mock (2.02±0.14) compared with cells transfected with pLKO.1-164 (1.06±0.02), pLKO.1-330 (1.42±0.16) and pLKO.1-711 (1.24±0.13) plasmids (Figure 8C).

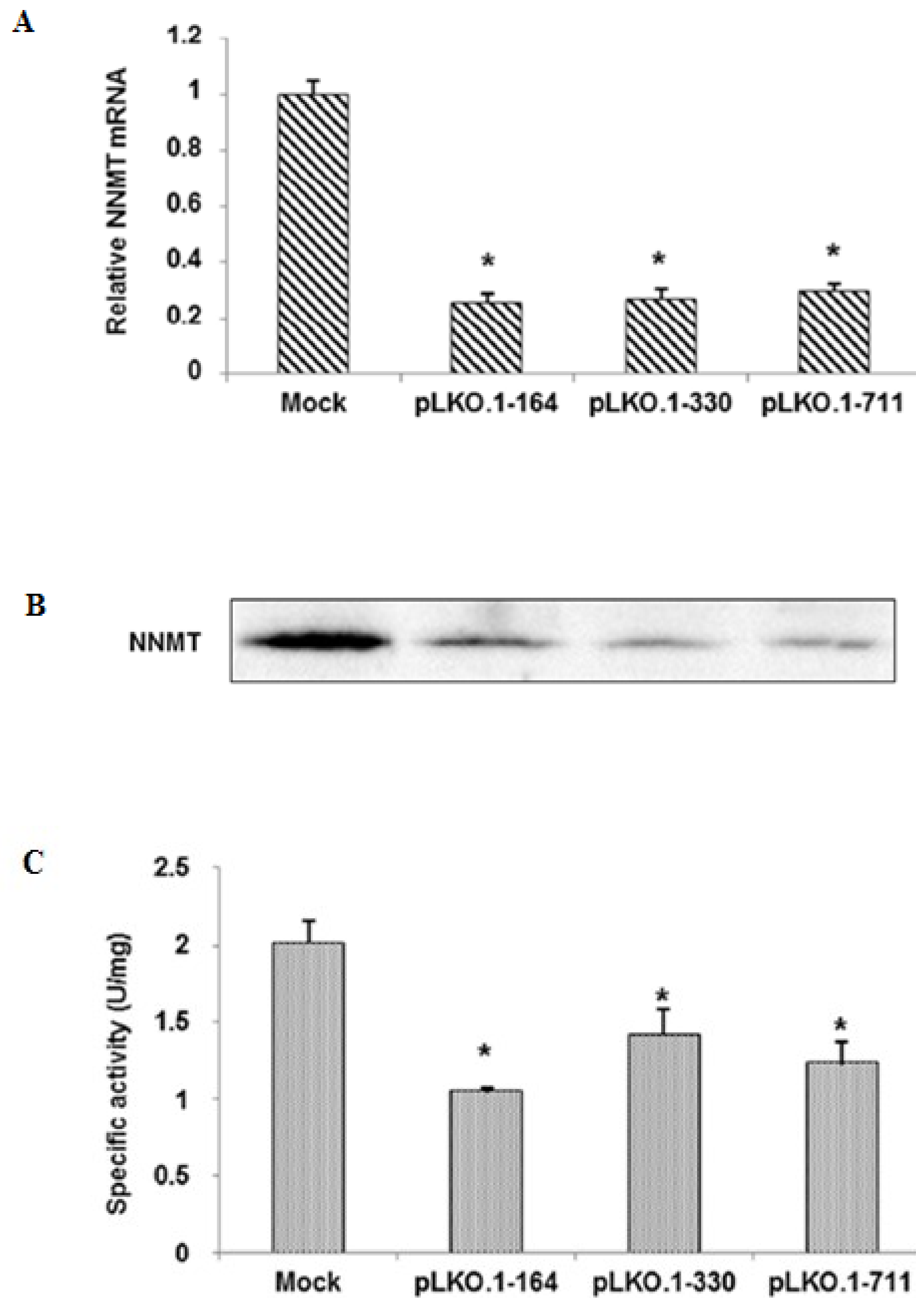

Figure 8. Evaluation of NNMT silencing. NNMT expression levels were analyzed in A549 cells transfected with 3 shRNA plasmids (pLKO.1-164, 1-330, and 1-711) or treated with transfection reagent only (mock), by Real-Time PCR (A), Western Blot (B), and enzyme assay (C). Values are reported as mean ± standard deviation (*$p < 0.05$).

3.1.2 Effect of NNMT silencing on cell proliferation

Cell viability was assessed in transfected and control A549 cells by MTT assay, in order to examine the biological effect associated with enzyme downregulation. As shown in figure 9, cell proliferation was significantly reduced in A549 transfected with all 3 shRNA plasmids compared to control cells. The results of MTT colorimetric assay were expressed as relative cell viability referred to control (absorbance at time zero and equal to 100%). Enzyme downregulation resulted in significantly ($p < 0.05$) decreased percentage values at all time points (24, 48 and 72 h).

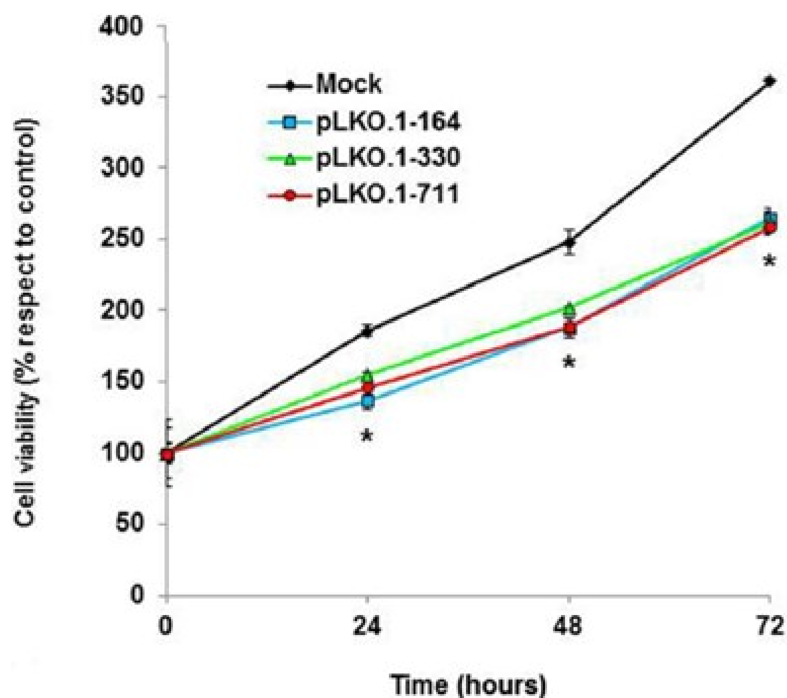

Figure 9. Cell proliferation was evaluated by MMT assay in transfected and control A549 cells after 24, 48, and 72 hours. Values are expressed as mean ± standard deviation (*p<0.05).

3.1.3 Effect of NNMT silencing on anchorage-independent cell growth

The effect of NNMT silencing on tumorigenesis *in vitro* was examined by monitoring anchorage-independent cell growth by soft-agar colony formation assay. After 30 days of incubation in semisolid culture medium, A549 cells transfected with pLK0.1-164 displayed colonies less numerous and smaller than those formed in control cells (Figure 10).

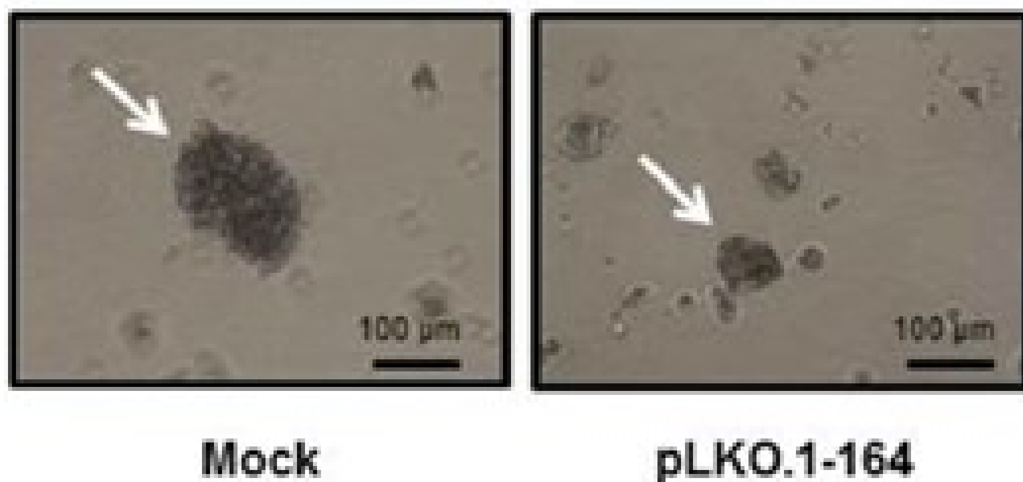

Figure 10. Anchorage-independent cell growth was assessed by soft-agar colony formation assay in transfected and control A549 cells after 30 days of incubation. Values are expressed as mean ± standard deviation (*$p<0.05$).

3.2 NNMT OVEREXPRESSION IN HSC-2 CELLS

3.2.1 NNMT cloning into pcDNA3 vector

Agarose gel electrophoresis of the amplified PCR product, obtained as described in the paragraph 2.2.2, revealed a single band of approximately 800 bp, corresponding to the human NNMT coding sequence (Figure 11).

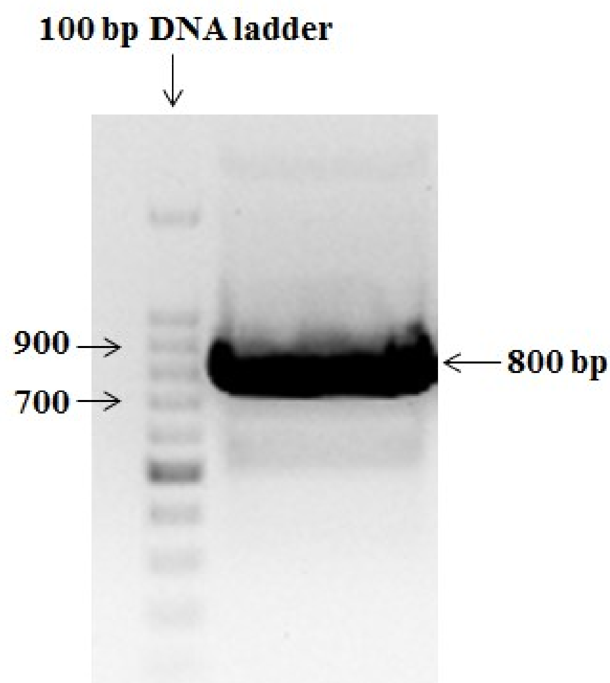

Figure 11. The amplification product of the human NNMT coding sequence.

After purification of the PCR product isolated from the agarose gel, insert and vector DNA were digested using BamHI and XhoI restriction enzymes. DNA fragments of the insert DNA and the linearized pcDNA3 vector were separated according to their size by agarose gel electrophoresis (Figure 12).

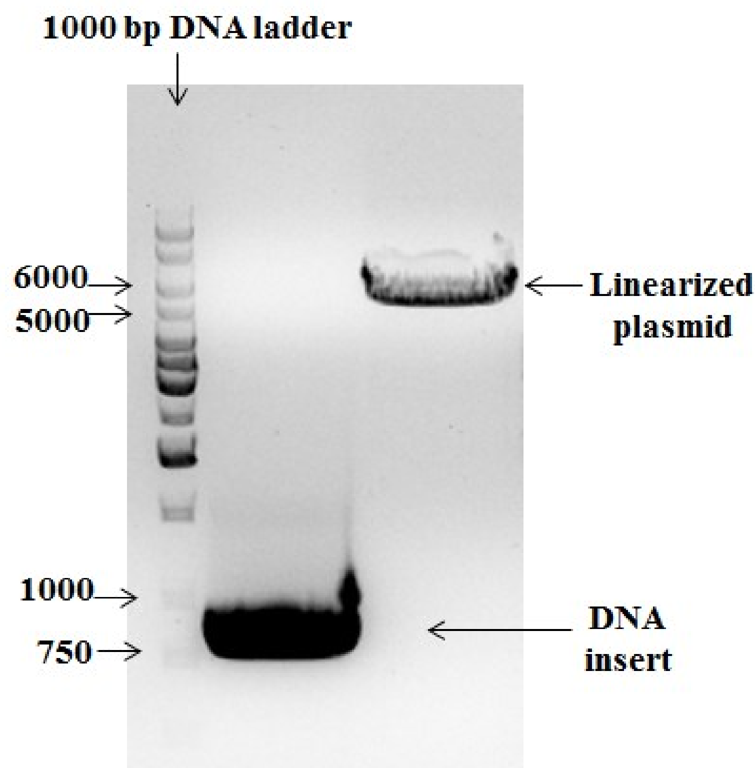

Figure 12. Separation of vector and insert DNA by agarose gel electrophoresis.

Recombinant plasmid, obtained through ligation between the insert and vector DNA, was used for transformation of E. coli JM109 competent cells. After transformation, bacterial cells were seeded onto a LB-agar plate containing 0.1 mg/ml ampicillin and nine of the grown clones were subjected to colony-PCR. Agarose gel electrophoresis of the amplification products confirmed the presence of the recombinant vector in bacterial cells (Figure 13).

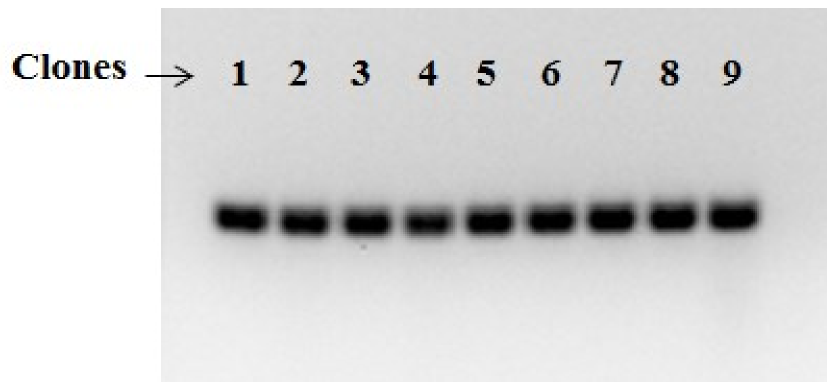

Figure 13. Agarose gel electrophoresis of amplification products obtained by colony-PCR performed on transformed bacterial clones.

After purification of the recombinant plasmid (pcDNA3-NNMT), results obtained from the analysis of the nucleotide sequence confirmed the presence of the NNMT insert within the pcDNA3 vector. Moreover, NNMT fragment did not contain any change in the nucleotide sequence possibly affecting the correct synthesis of the recombinant protein.

3.2.2 Efficiency of NNMT overexpression

HSC-2 cell line was transiently transfected with the NNMT expression vector (pcDNA3-NNMT), and control cells were transfected with the empty vector (pcDNA3) or treated with transfection reagent only (mock), as described in the paragraph 2.2.3. Forty-eight hours after transfection cells were harvested. To evaluate enzyme overexpression, NNMT mRNA, protein and catalytic activity levels were analyzed by Real-Time PCR, Western blot and enzyme assay, respectively.

Real-Time PCR showed a significant upregulation of NNMT mRNA level in cells transfected with pcDNA3-NNMT plasmid (10087 ± 1495) compared with pcDNA3-treated (0.78 ± 0.08) and mock (1.00 ± 0.13) cells (Figure 14A).

Western blot analysis revealed a single band at approximately 30 KDa, corresponding to the known molecular mass of NNMT, in HSC-2 cells treated with pcDNA3-NNMT, while a very weak NNMT band was detected in control cells, confirming NNMT overexpression at protein level (Figure 14B).

Consistent with results of Real-Time PCR and Western blot analysis, the levels of NNMT specific activity, expressed in U/mg protein, were particularly higher in cells transfected with the pcDNA3-NNMT (8.59 ± 0.46), as compared with controls in which the activity was found to be below the detection limit of the assay. (Figure 14C).

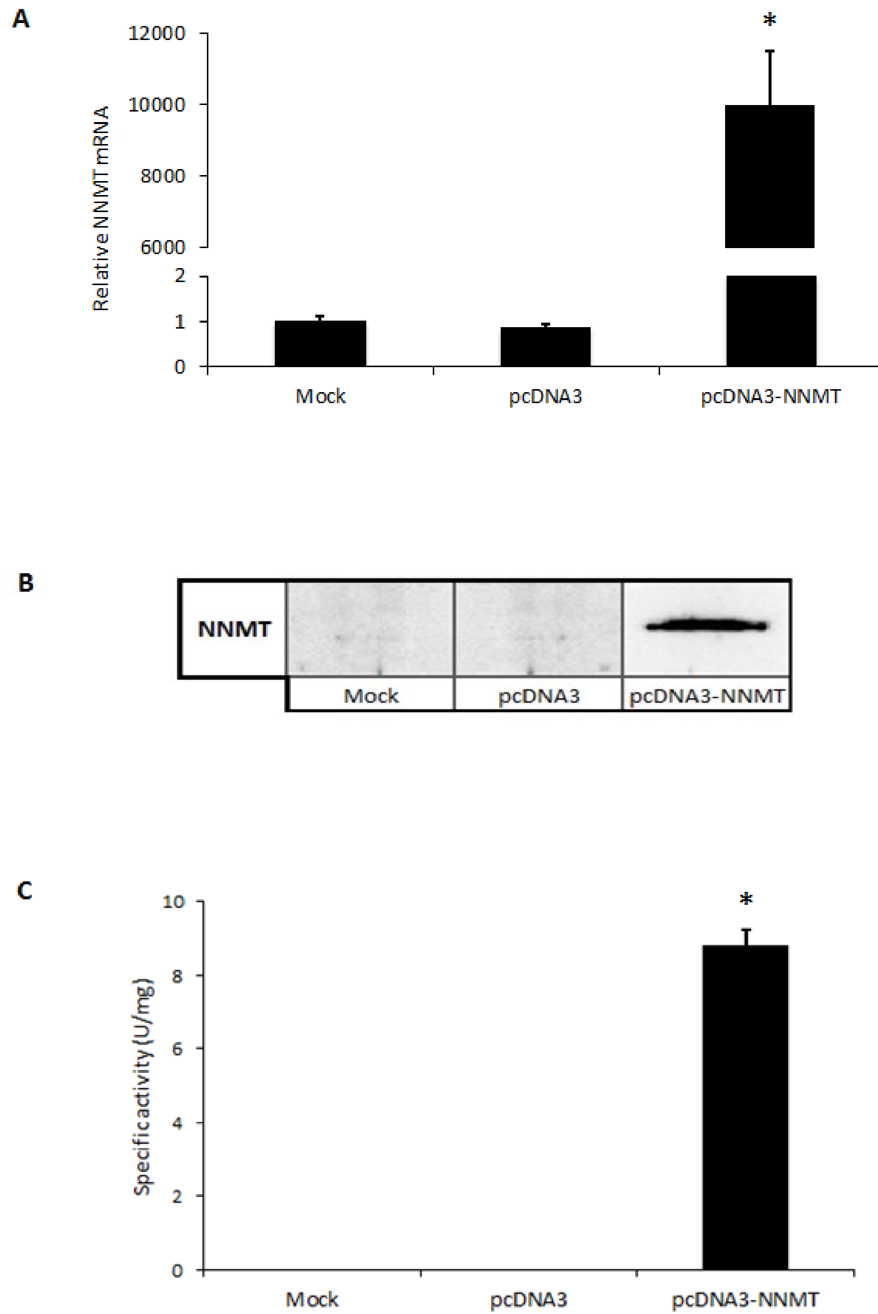

Figure 14. NNMT overexpression. NNMT mRNA levels were evaluated by Real-Time PCR in transfected (pcDNA3 and pcDNA3-NNMT) compared with mock cells (A). Protein lysates were analyzed by Western blot to measure NNMT protein levels (B). NNMT specific activity was determined using an HPLC-based method (C). All values are expressed as mean ± standard deviation (*$p<0.05$).

3.2.3 Effect of NNMT overexpression on cell proliferation

To analyze the biological effect associated with enzyme upregulation, pcDNA3-NNMT vector was used to transfect HSC-2 cells, and the effect of NNMT overexpression on cell proliferation was evaluated by MTT assay. As shown in Figure 15, pcDNA3-NNMT plasmid was able to increase cell growth of HSC-2 cells compared with controls. The results of MTT colorimetric assay were expressed as relative cell viability referred to control. Enzyme upregulation led to a significant ($p < 0.05$) increase of percentage value at 72h time point.

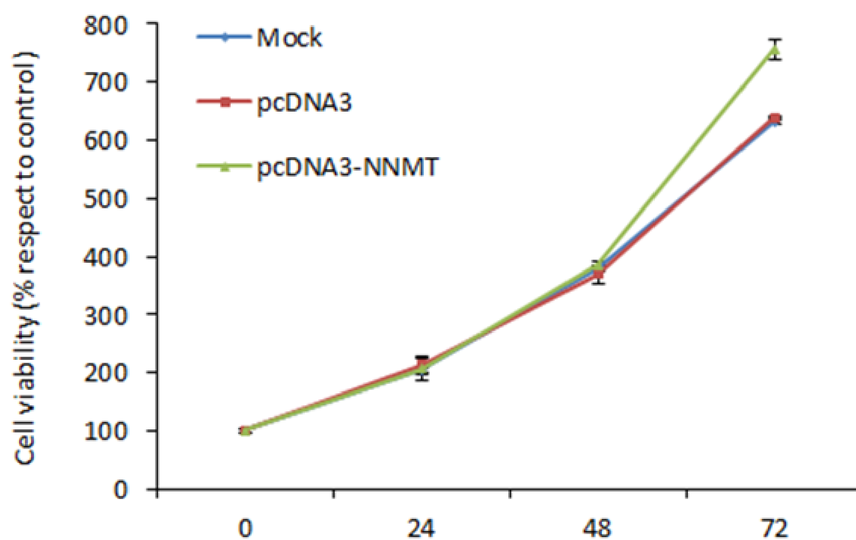

Figure 15. The effect of NNMT overexpression on cell proliferation was assessed by MTT assay. Cell growth was evaluated in mock and transfected HSC-2 cells after 24, 48 and 72 hours of incubation. All values are expressed as mean ± standard deviation ($*p<0.05$).

3.2.4 Effect of NNMT upregulation on β-catenin, survivin, and Ki-67 expression

To explore the potential involvement of the enzyme in cellular pathways, such as apoptosis, cell proliferation and cell signaling, the mRNA expression levels of β-catenin, Ki-67, and survivin isoforms were analyzed in HSC-2 cells transfected with pcDNA3-NNMT, with the empty vector (pcDNA3), or treated with transfection reagent only (mock). Agarose gel electrophoresis showed a single and strong stained band obtained from the amplification of β-catenin, Ki-67, survivin main isoform, survivin ΔEx3, and survivin 2B. On the contrary, a very weak band was detected for survivin 2α and survivin 3B (Figure 16).

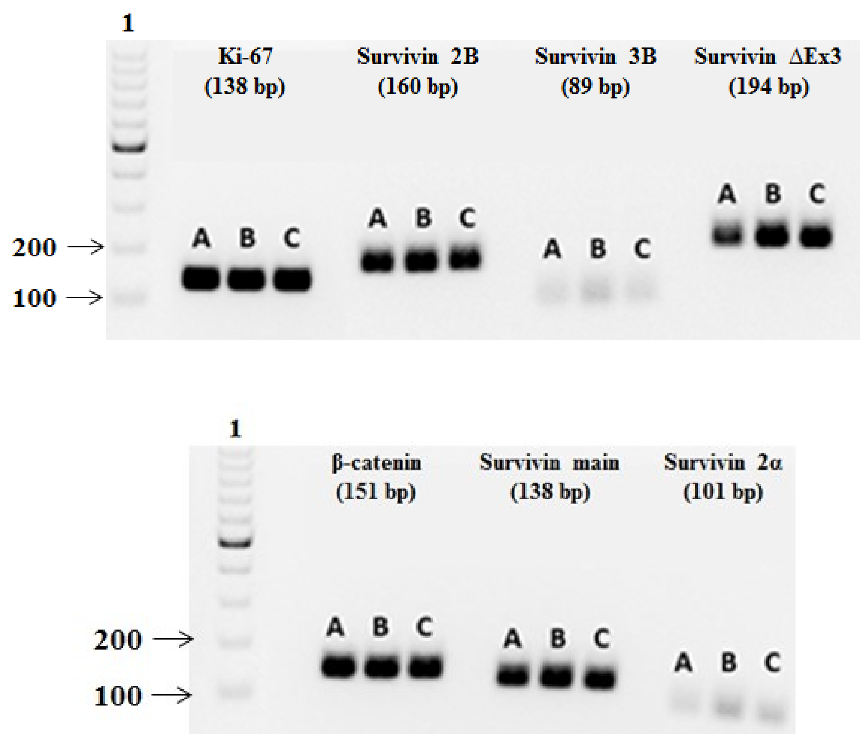

Figure 16. Agarose gel electrophoresis of PCR products. DNA ladder (1), mock (A), pcDNA3 (B), and pcDNA3-NNMT (C).

Results obtained by Real-Time PCR are shown in Figure 17. No significant differences were detected in both β-catenin and survivin main isoform expression levels between HSC-2 transfected with pcDNA3-NNMT and controls. On the contrary, survivin ΔEx3 levels were significantly ($p < 0.05$) higher in NNMT overexpressing cells (pcDNA3-NNMT) compared with those detected in HSC-2 transfected with empty vector (pcDNA3) or treated with transfection reagent only (mock). Survivin 2B isoform was overexpressed in HSC-2 transfected with pcDNA3-NNMT compared with those treated with pcDNA3. However, its expression levels did not differ significantly between pcDNA3-NNMT-treated and mock cells. Ki-67 expression levels were higher in cells transfected with pcDNA3-NNMT compared with mock, but no significant differences were shown when compared with pcDNA3-treated cells.

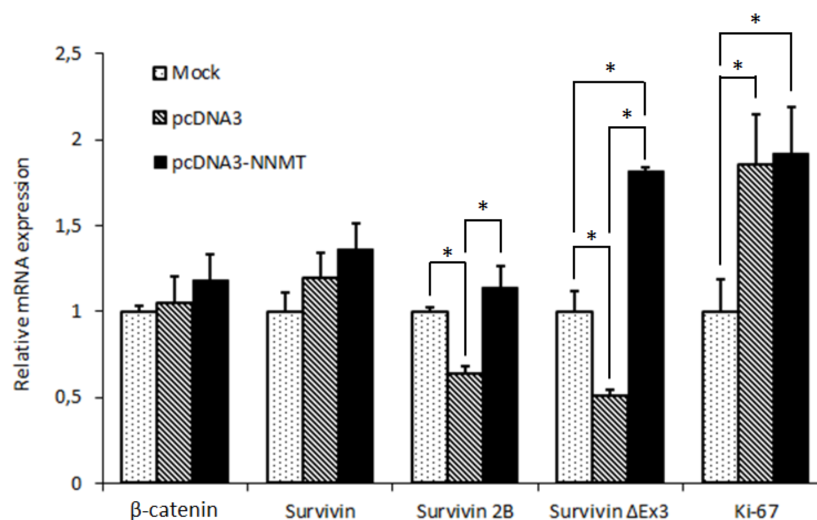

Figure 17. Expression levels of β-catenin, survivin isoforms, and Ki-67. Real-Time PCR was used to evaluate mRNA levels of β-catenin, survivin isoforms, and Ki-67 in HSC-2 cells transfected with pcDNA3-NNMT or pcDNA3, or treated with transfection reagent only (mock). All values are expressed as mean ± standard deviation (*$p<0.05$).

4. DISCUSSION AND CONCLUSIONS

Lung cancer is the leading cause of tumor-related death worldwide and is classified into two main histological subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which represent 80% of all lung cancer cases. The 5-year survival rate is approximately 50% for patients with early stage NSCLC, while survival declines to approximately 4% in patients with advanced stage disease. The majority of lung cancers are diagnosed at a late stage, with locally advanced or metastatic disease, while only 15% of cases are diagnosed at an early stage. Despite progress in the therapeutic strategies, the prognosis of patients affected with lung cancer remains poor. Thus, the identification of reliable biomarkers to early diagnose and monitor the progression of the disease, as well as to evaluate the efficacy of a therapeutic approach is urgently needed [141].

The present work is focused on nicotinamide N-methyltransferase (NNMT), a phase II metabolizing enzyme which catalyzes the N-methylation of nicotinamide, pyridines and other structural analogs, playing an important role in the biotransformation and detoxification of many xenobiotic compounds [22]. The enzyme is mainly expressed in the liver, while reduced levels were detected in the kidney, skeletal muscle, lung, heart, placenta, bladder and brain [38, 82]. NNMT upregulation has been reported in several neoplasms, including glioblastoma [93], gastric cancer [107], thyroid cancer [95], colorectal cancer [103], RCC [110], and bladder cancer [121].

In lung cancer, elevated serum NNMT level was detected in NSCLC patients compared with both healthy donors and patients with COPD [123]. Moreover, in a recent study NNMT expression levels were analyzed in tumor, tumor-adjacent, and surrounding tissue samples from patients with NSCLC by Real-Time PCR, western blot and catalytic activity assay. NNMT mRNA, protein, and activity levels were significantly higher in tumor compared with both tumor-adjacent and surrounding tissue [124].

Subsequent immunohistochemical analyses confirmed results previously obtained, revealing an upregulation of NNMT protein in NSCLC tissue samples [125]. While the above studies have shown that NNMT is overexpressed in lung cancer, none of these have explored the biological effects associated with enzyme upregulation. In the present work, in order to investigate whether NNMT may have a role in the tumorigenesis of lung cancer, the cellular effects of NNMT silencing were examined in human lung cancer cell line A549. Plasmid vectors expressing shRNAs targeted to different regions of NNMT gene sequence efficiently led to NNMT downregulation in A549 cells. The effects of NNMT knockdown on cell proliferation and anchorage-independent cell growth were evaluated by MTT and soft agar colony formation assays, respectively. NNMT downregulation significantly inhibited cell proliferation and decreased colony formation ability on soft agar, suggesting an important involvement of the enzyme in lung tumorigenesis.

In order to investigate the role of NNMT in cancer cell metabolism, subsequent analysis were performed exploring the cellular effects of NNMT overexpression in a human oral squamous cell carcinoma cell line.

OSCC is the most common malignancies of oral cavity. Despite progress in therapeutic strategies, the 5-year survival rate for OSCC has not improved over the past three decades, remaining slightly below 50%. The poor prognosis of patients with oral cancer is primarily due to delayed diagnosis, which leads the patients to be treated when the tumor has reached an advanced stage [142]. Thus, the study of the molecular mechanisms involved in carcinogenesis is necessary to identify new markers suitable for early diagnosis and prognosis, as well as to identify new targets for molecular-based treatments.

A number of studies have explored the potential involvement of NNMT in oral squamous cell carcinoma. Elevated NNMT expression were detected in tumor tissues compared with normal mucosa, and NNMT mRNA levels were inversely correlated with lymph node metastasis, pT and pathological stage [128]. In addition to the potential role of the enzyme as a prognostic factor, higher NNMT expression levels were shown in salivary sample obtained from patients with OSCC compared to those detected in specimens from healthy subjects, suggesting that NNMT could serve as a biomarker for early and non-invasive diagnosis of oral cancer [130].

In a recent study, NNMT levels were evaluated in several human oral cancer cell lines and the NNMT shRNA-mediated silencing in PE/CA-PJ15 cells led to a significant reduction of cell growth *in vitro* and tumorigenicity *in vivo* [132]. In the present work, the effect of NNMT overexpression was evaluated in the human oral cancer cell line HSC-2. This cell line displayed a very low endogenous NNMT expression, thus representing a suitable model for this study. NNMT Overexpression increased cell proliferation in HSC-2 cells, confirming that the enzyme plays an important role in oral cancer cell metabolism.

Taken together, results showed in this work are in line with those obtained in other studies that explored the involvement of NNMT in several cancers and non-neoplastic diseases.

Recent studies reported that NNMT downregulation in the human breast cancer cell lines Bcap-37 and MDAMB-231 significantly decreased cell growth *in vitro* and *in vivo*, increased ROS production and induced apoptosis via the mitochondria-mediated pathway [98], while overexpression of the enzyme in SW480 colorectal cancer cell line promoted tumorigenicity *in vitro* and *in vivo*, inhibited apoptosis, increased intracellular ATP levels and decreased ROS production [104].

Upregulation of the enzyme in skeletal myoblasts led to a significant increase in cell proliferation and migration, as well as reduced cell death induced by exposing myoblasts to ROS. In this light, enhanced NNMT expression may represent a strategy to react to oxidative stress, leading to increased myogenesis and protection against oxidative stress [72]. The cellular effects of NNMT overexpression was also investigated in the human neuroblastoma cell line SH-SY5Y, which lacks of endogenous NNMT. NNMT expression significantly increased cell viability, Complex I activity and intracellular ATP content. In addition, both NNMT expression and N1-methylnicotinamide treatment protected SH-SY5Y cells from the toxicity of CxI inhibitors MPP+ and rotenone, suggesting a possible cytoprotective role of the enzyme [88].

Several studies demonstrated that NNMT is involved in cellular invasion. Indeed, NNMT silencing significantly reduced cell migration and proliferation in human bladder cancer cells [120], and decreased invasive capacity of ccRCC cells [115]. In addition, NNMT downregulation significantly reduced cell proliferation, migration and invasion capacity in the human pancreatic carcinoma cell line PANC-1 [101], suggesting a potential role of the enzyme in tumor progression.

As already mentioned, NNMT catalyzes the N-methylation of nicotinamide to N1-methylnicotinamide, which is excreted into urine, and is the only enzyme known to utilize nicotinamide as a methyl acceptor substrate, playing a fundamental role in the catabolism of this compound. Therefore, NNMT could regulate nicotinamide intracellular levels, and its overexpression may affect cellular process in which nicotinamide appears to be involved. Nicotinamide is a precursor for the synthesis of nicotinamide adenine dinucleotide (NAD), which plays a key role in the energy production through redox reactions as well as participates in several non-redox

mechanisms. In this regard, nicotinamide has been proposed as a strong inhibitor of enzymes such as poly ADP-ribose polymerases (PARPs) [23], which play an important role in DNA damage repair, and histone deacetylases (sirtuins), involved in the regulation of gene expression, longevity and genome stability [25, 26]. Studies conducted in a bladder carcinoma cell line [117] and in tumorigenic mesenchymal CSC clones [118], have shown a correlation between the resistance to radiotherapy and NNMT expression levels. In the light of the above considerations, high NNMT activity may reduce PARP inhibition exerted by nicotinamide, resulting in elevated radiation resistance. In a recent study focused on chronic lymphocytic leukemia (CLL) cells, nicotinamide treatment inhibited the endogenous SIRT1 deacetylase activity, leading to a block of proliferation and induction of apoptosis. Furthermore, the exposure to nicotinamide enhanced the apoptotic effects of the DNA damaging agent etoposide in a p53-dependent manner [143]. In this regard, NNMT activity, lowering nicotinamide intracellular levels, could play an important role in both tumor growth and chemoresistance.

NNMT catalyzes the transfer of the methyl group from S-adenosyl-L-methionine (SAM) to nicotinamide, generating S-adenosyl-L-homocysteine (SAH) and N1-methylnicotinamide. Therefore, it is conceivable that alterations in NNMT expression could lead to variations of methylation potential of cancer cells, defined as the ratio SAM:SAH [144]. In this light, NNMT was shown to regulate the protein methylation state of tumor cells by altering cellular SAM/SAH ratio. As results, NNMT affected the methylation of histones and cancer-related proteins, leading to the activation of protumorigenic factors [134].

NNMT catalytic activity also plays a critical role in the regulation of endogenous levels of N1-methylnicotinamide, a compound which displayed vasoprotective [145],

gastroprotective [146], anti-inflammatory [64], neuroprotective [89], and anti-thrombotic [62] properties. In this regard, it could be interesting to speculate whether N1-methylnicotinamide exerts a cytoprotective effect against antineoplastic agents in cancer cells. Indeed, the upregulation of the enzyme observed in tumors, which lead to elevated N1-methylnicotinamide intracellular levels, could confer an adaptive advantage to cancer cells.

NNMT overexpression may also affect the metabolism of anticancer compounds. Since the enzyme shows a low substrate specificity, its methyltransferase activity could alter the efficacy and/or the elimination of chemotherapeutic drugs.

In the present work, in order to further examine the involvement of the enzyme in cancer cell metabolism, the effect of NNMT on apoptosis, cell proliferation, and cell signaling was investigated at molecular level, by exploring the expression of β-catenin, survivin isoforms, and Ki-67 in HSC-2 transfected with pcDNA3-NNMT and control cells. Enzyme upregulation did not alter the expression level of β-catenin, Ki-67, survivin 2B and survivin main isoform. On the contrary, survivin ΔEx3 isoform levels were significantly higher in NNMT overexpressing cells compared with controls.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family, containing a single baculovirus IAP repeat (BIR) domain, and is involved in cell cycle progression and control of apoptosis [147]. Survivin overexpression has been reported in several human tumors, where it has been associated with tumor proliferative activity, poor overall survival, as well as resistance to chemotherapy and radiotherapy [148-150], thus suggesting its potential involvement in tumor progression. The survivin gene consists of 4 exons encoding for a 16,5 kDa protein. In addition, other five alternative splice variants of survivin have been identified, including survivin-ΔEx3 (lacking exon 3 and containing part of 3'-UTR), survivin-2B (including part of intron 2, called exon 2b),

survivin-3B (including part of intron 3, called exon 3B, containing a stop codon), survivin-2α, and survivin-3α. Main survivin isoform can heterodimerize with its splice variants, and it has been suggested that heterodimers formation can lead to the different biological functions observed in tumor cells [151]. Of particular interest is survivin-ΔEx3, an antiapoptotic protein that it has been reported to be upregulated in oral cancer [152] as well as in other malignancies [153]. In several human cancers, survivin-ΔEx3 has been associated with advanced tumor stage, tumor aggressiveness, as well as poor prognosis [154, 155], suggesting a role for this splice variant in oral tumorigenesis.

In conclusion, results obtained demonstrated that NNMT plays an important role in proliferation and tumorigenic capacity of both lung and oral cancer cells, thus representing a potential molecular target for an effective anti-cancer therapy. Despite experimental evidences demonstrate that NNMT is involved in tumorigenesis, further studies are required to deeply understand the molecular mechanisms affected by the enzyme in cancer cells. Data obtained also showed a possible correlation between NNMT and survivin-ΔEx3 expression levels, suggesting a potential role of the enzyme in the regulation of apoptosis.

5. LIST OF ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ADC	Adenocarcinoma
ADP	Adenosine 5'-diphosphate
ALL	Acute lymphoblastic leukemia
ALK	Anaplastic lymphoma kinase
ART	Mono-ADP-ribosyltransferase
ATP	Adenosine 5'-triphosphate
cADPR	Cyclic adenosine 5'-diphosphate-ribose
ccRCC	Clear cell renal cell carcinoma
CEA	Carcinoembryonic Antigen
cDNA	Complementary DNA
COMT	Catechol-O-methyltransferase
ConA	ConcanavalinA
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CXR	Chest x-rays
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ETS	Environmental tobacco smoke
GBM	Glioblastoma Multiforme
HNF-1β	Hepatocyte nuclear factor beta 1
HNMT	Histamine N-methyltransferase
HPLC	High-performance liquid chromatography
ICSI	Intracytoplasmic sperm injection
IL-6	Interleukin-6

LC	Large cell carcinoma
LDCT	Low-dose computed tomography
LIF	Leukemia Inhibitory Factor
LOH	Loss of heterozygosity
KRAS	Kirsten rat sarcoma viral oncogene homolog
MNA	N1-methylnicotinamide
MPP+	1-methyl-4-phenylpyridinium ion
mRNA	Messenger RNA
MTHFR	Methylene tetrahydrofolate reductase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAADP	Nicotinic acid adenine dinucleotide phosphate
NAD+	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NNMT	Nicotinamide N-methyltransferase
NSCLC	Non-small cell lung cancer
OSCC	Oral Squamous Cell Carcinoma
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP-ribose polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
POMT	Phenol O-methyltransferase
PTC	Proximal tubular cell
PVDF	Polyvinylidene fluoride
RB	Retinoblastoma protein
ROS	Reactive oxygen species

RNA	Ribonucleic acid
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SCC	Squamous cell carcinoma
SCLC	Small-cell lung cancer
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
Sir2	Sirtuin
SNP	Single nucleotide polymorphisms
STAT3	Signal Trasducer and Activator of transcription 3
TAE	Tris-acetate-EDTA
TC	Computed tomography
TGF-β1	Transforming growth factor beta 1
TMT	Thiol methyltransferase
TPMT	Thiopurine methyltransferase
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
UC	Urothelial carcinoma
WHO	World Health Organization

6. REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136(5):E359-86.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65(2):87-108.
3. Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C, Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch R, Jemal A, Ward E. Cancer treatment and survivorship statistics, 2012. CA Cancer J Clin 2012; 62(4):220-41.
4. Kasper DL, Fauci AS, Hauser S, Longo D, Jameson JL, Loscalzo J. Harrison’s Principles of Internal Medicine. 19th Edition.
5. Watanabe M. Smoking: additional burden on aging and death. Genes Environ 2016; 38:3.
6. Brennan P, Hainaut P, Boffetta P. Genetics of lung-cancer susceptibility. Lancet Oncol 2011; 12(4):399-408.
7. Minna JD, Roth JA, Gazdar AF. Focus on lung cancer. Cancer Cell. 2002; 1(1):49-52. Review.
8. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC Cancer Staging Manual and the future of TNM. Ann Surg Oncol 2010; 17(6):1471-1474.
9. Ganti AK, Zhen W, Kessinger A. Limited-stage small-cell lung cancer: therapeutic options. Oncology 2007; 21(3):303-312.

10. Li ZY, Luo L, Hu YH, Chen H, Den YK, Tang L, Liu B, Liu D, Zhang XY. Lung cancer screening: a systematic review of clinical practice guidelines. Int J Clin Pract 2016; 70(1):20-30.
11. Messadi DV. Diagnostic aids for detection of oral precancerous conditions. Int J Oral Sci 2013; 5(2):59-65.
12. Kumar M, Nanavati R, Modi TG, Dobariya C. Oral cancer: Etiology and risk factors: A review. J Cancer Res Ther 2016; 12(2):458-463.
13. Kang CJ, Liao CT, Hsueh C, Lee LY, Lin CY, Fan KH, Wang HM, Huang SF, Chen IH, Ng SH, Tsao CK, Huang YC, Yen TC. Outcome analysis of patients with well-differentiated oral cavity squamous cell carcinoma. Oral Oncol 2011; 47(11):1085-1091.
14. Crescenzi D, Laus M, Radici M, Croce A. TNM classification of the oral cavity carcinomas: some suggested modifications. Otolaryngol Pol. 2015; 69(4):18-27.
15. Villa A, Villa C, Abati S. Oral cancer and oral erythroplakia: an update and implication for clinicians. Aust Dent J 2011; 56(3):253-256.
16. Carreras-Torras C, Gay-Escoda C. Techniques for early diagnosis of oral squamous cell carcinoma: Systematic review. Med Oral Patol Oral Cir Bucal 2015; 20(3):e305-315.
17. Rashid A, Warnakulasuriya S. The use of light-based (optical) detection systems as adjuncts in the detection of oral cancer and oral potentially malignant disorders: a systematic review. J Oral Pathol Med 2015; 44(5):307-328.
18. Huang SH, O'Sullivan B. Oral cancer: Cuncurrent role of radiotherapy and chemotherapy. Med Oral Patol Oral Cir Bucal 2013; 18(2):e233-240.

19. Lefebvre JL. Current clinical outcomes demand new treatment options for SCCHN. Ann Oncol 2005; 16 Suppl 6:vi7-vi12.
20. Meyer UA. Overview of enzymes of drug metabolism. J Pharmacokinet Biopharm 1996; 24(5):449-459.
21. Weinshilboum RM. Human pharmacogenetics of methyl conjugation. Fed Proc 1984; 43(8):2303-2307.
22. Weinshilboum RM. Methyltransferase pharmacogenetics. Pharmacol Ther 1989; 43(1):77-90.
23. Ueda K, Hayaishi O. ADP-ribosylation. Annu Rev Biochem 1985; 54:73-100.
24. Clapper DL, Walseth TF, Dargie PJ, Lee HC. Pyridine nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol trisphosphate. J Biol Chem 1987; 262(20):9561-9568.
25. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 2000; 403(6771):795-800.
26. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 2001; 107(2):149-159.
27. Bechgaard H, Jespersen S. GI absorption of niacin in humans. J Pharm Sci 1977; 66(6):871-872.
28. Bender DA. Biochemistry of tryptophan in health and disease. Mol Aspects Med 1983; 6(2):101-197.
29. Horwitt MK, Harper AE, Henderson LM. Niacin-tryptophan relationships for evaluating niacin equivalents. Am J Clin Nutr 1981; 34(3):423-427.

30. Bernofsky C. Physiology aspects of pyridine nucleotide regulation in mammals. Mol Cell Biochem 1980; 33(3):135-143.
31. D'Souza J, Caldwell J, Smith RL. Species variations in the N-methylation and quaternization of [14C]pyridine. Xenobiotica 1980; 10(2):151-157.
32. Okamoto H, Ishikawa A, Yoshitake Y, Kodama N, Nishimuta M, Fukuwatari T, Shibata K. Diurnal variations in human urinary excretion of nicotinamide catabolites: effects of stress on the metabolism of nicotinamide. Am J Clin Nutr 2003; 77(2):406-410.
33. Crooks PA, Godin CS, Damani LA, Ansher SS, Jakoby WB. Formation of quaternary amines by N-methylation of azaheterocycles with homogeneous amine N-methyltransferases. Biochem Pharmacol 1988; 37(9):1673-1677.
34. Sano A, Endo N, Takitani S. Fluorometric assay of rat tissue N-methyltransferases with nicotinamide and four isomeric methylnicotinamides. Chem Pharm Bull (Tokyo) 1992; 40(1):153-156.
35. Rini J, Szumlanski C, Guerciolini R, Weinshilboum RM. Human liver nicotinamide N-methyltransferase: ion-pairing radiochemical assay, biochemical properties and individual variation. Clin Chim Acta 1990; 186(3):359-374.
36. Weinshilboum RM, Raymond FA. Inheritance of low erythrocyte catechol-o-methyltransferase activity in man. Am J Hum Genet 1977; 29(2):125-135.
37. Weinshilboum RM, Sladek SL. Mercaptopurine pharmacogenetics: monogenic inheritance of erythrocyte thiopurine methyltransferase activity. Am J Hum Genet 1980; 32(5):651-662.
38. Aksoy S, Szumlanski CL, Weinshilboum RM. Human liver nicotinamide N-methyltransferase. cDNA cloning, expression, and biochemical characterization. J Biol Chem 1994; 269(20):14835-14840.

39. Aksoy S, Brandriff BF, Ward A, Little PF, Weinshilboum RM. Human nicotinamide N-methyltransferase gene: molecular cloning, structural characterization and chromosomal localization. Genomics 1995; 29(3):555-561.
40. Xu J, Capezzone M, Xu X, Hershman JM. Activation of nicotinamide N-methyltransferase gene promoter by hepatocyte nuclear factor-1beta in human papillary thyroid cancer cells. Mol Endocrinol 2005; 19(2):527-539.
41. Xu J, Hershman JM. Histone deacetylase inhibitor depsipeptide represses nicotinamide N-methyltransferase and hepatocyte nuclear factor-1beta gene expression in human papillary thyroid cancer cells. Thyroid 2006; 16(2):151-160.
42. Tomida M, Ohtake H, Yokota T, Kobayashi Y, Kurosumi M. Stat3 up-regulates expression of nicotinamide N-methyltransferase in human cancer cells. J Cancer Res Clin Oncol 2008; 134(5):551-559.
43. Nabokikh A, Ilhan A, Bilban M, Gartner W, Vila G, Niederle B, Nielsen JH, Wagner O, Base W, Luger A, Wagner L. Reduced TGF-beta1 expression and its target genes in human insulinomas. Exp Clin Endocrinol Diabetes 2007; 115(10):674-682.
44. Peng Y, Sartini D, Pozzi V, Wilk D, Emanuelli M, Yee VC. Structural basis of substrate recognition in human nicotinamide N-methyltransferase. Biochemistry 2011; 50(36):7800-7808.
45. Smith ML, Burnett D, Bennett P, Waring RH, Brown HM, Williams AC, Ramsden DB. A direct correlation between nicotinamide N-methyltransferase activity and protein levels in human liver cytosol. Biochim Biophys Acta 1998; 1442(2-3):238-244.

46. Saito S, Iida A, Sekine A, Miura Y, Sakamoto T, Ogawa C, Kawauchi S, Higuchi S, Nakamura Y. Identification of 197 genetic variations in six human methyltranferase genes in the Japanese population. J Hum Genet 2001; 46(9):529-537.
47. Suoto JC, Blanco-Vaca F, Soria JM, Buil A, Almasy L, Ordoñez-Llanos J, Martìn-Campos JM, Lathrop M, Stone W, Blangero J, Fontcuberta J. A genomewide exploration suggests a new candidate gene at chromosome 11q23 as the major determinant of plasma homocysteine levels: results from the GAIT project. Am J Hum Genet 2005; 76(6):925-933.
48. van Driel LM, Smedts HP, Helbing WA, Isaacs A, Lindemans J, Uitterlinden AG, van Duijn CM, de Vries JH, Steegers EA, Steegers-Theunissen RP. Eight-fold increased risk for congenital heart defects in children carrying the nicotinamide N-methyltransferase polymorphism and exposed to medicines and low nicotinamide. Eur Heart J 2008; 29(11):1424-1431.
49. Lu W, Zhu H, Wen S, Yang W, Shaw GM, Lammer EJ, Finnell RH. Nicotinamide N-methyl transferase (NNMT) gene polymorphisms and risk for spina bifida. Birth Defects Res A Clin Mol Teratol 2008; 82(10):670-675.
50. Giusti B, Sestini I, Saracini C, Sticchi E, Bolli P, Magi A, Gori AM, Marcucci R, Gensini GF, Abbate R. High-throughput multiplex single-nucleotide polymorphism (SNP) analysis in genes involved in methionine metabolism. Biochem Genet 2008; 46(7-8):406-423.
51. Giusti B, Saracini C, Bolli P, Magi A, Sestini I, Sticchi E, Pratesi G, Pulli R, Pratesi C, Abbate R. Genetic analysis of 56 polymorphisms in 17 genes involved in methionine metabolism in patients with abdominal aortic aneurysm. Med Genet 2008; 45(11):721-730.

52. Giusti B, Saracini C, Bolli P, Magi A, Martinelli I, Peyvandi F, Rasura M, Volpe M, Lotta LA, Rubattu S, Mannucci PM, Abbate R. Early-onset ischaemic stroke: analysis of 58 polymorphisms in 17 genes involved in methionine metabolism. Thromb Haemost 2010; 104(2):231-242.
53. De Jonge R, Tissing WJ, Hooijberg JH, Jansen G, Kaspers GJ, Lindemans J, Peters GJ, Pieters R. Polymorphisms in folate-related genes and risk of pediatric acute lymphoblastic leukemia. Blood 2009; 113(10):2284-2289.
54. Bromberg A, Lerer E, Udawela M, Scarr E, Dean B, Belmaker RH, Ebstein R, Agam G. Nicotinamide-N-methyltransferase (NNMT) in schizophrenia: genetic association and decreased frontal cortex mRNA levels. Int J Neuropsychopharmacol 2012; 15(6):727-737.
55. Wang GX, Zhang Y, Lv ZW, Sun M, Wu D, Chen XY, Wu YM. Female specific association between NNMT gene and schizophrenia in a Han Chinese population. Int J Med Sci 2014; 11(12):1234-1239.
56. Sazci A, Ozel MD, Ergul E, Aygun C. Association of nicotinamide-N-methyltransferase gene rs694539 variant with patients with nonalcoholic steatohepatitis. Genet Test Mol Biomarkers 2013; 17(11):849-853.
57. Sazci A, Ozel MD, Ergul E, Onder ME. Association of nicotinamide-N-methyltransferase (NNMT) gene rs694539 variant with bipolar disorder. Gene 2013; 532(2):272-275.
58. Sazci G, Sazci B, Sazci A, Idrisoglu HA. Association of Nicotinamide-N-Methyltransferase Gene rs694539 Variant with Epilepsy. Mol Neurobiol; 201653(6):4197-4200.
59. Cuomo R, Dattilo M, Pumpo R, Capuano G, Boselli L, Budillon G. Nicotinamide methylation in patients with cirrhosis. J Hepatol 1994; 20(1):138-142.

60. Bryniarski K, Biedron R, Jakubowski A, Chlopicki S, Marcinkiewicz J. Anti-inflammatory effect of 1-methylnicotinamide in contact hypersensitivity to oxazolone in mice; involvement of prostacyclin. Eur J Pharmacol 2008; 578(2-3):332-338.
61. Gebicki J, Sysa-Jedrzejowska A, Adamus J, Woźniacka A, Rybak M, Zielonka J. 1-Methylnicotinamide: a potent anti-inflammatory agent of vitamin origin. Pol J Pharmacol 2003; 55(1):109-112.
62. Chlopicki S, Swies J, Mogielnicki A, Buczko W, Bartus M, Lomnicka M, Adamus J, Gebicki J. 1-Methylnicotinamide (MNA), a primary metabolite of nicotinamide, exerts anti-thrombotic activity mediated by a cyclooxygenase-2/prostacyclin pathway. Br J Pharmacol 2007; 152(2):230-239.
63. Mateuszuk Ł, Khomich TI, Słomińska E, Gajda M, Wójcik L, Łomnicka M, Gwóźdź P, Chłopicki S. Activation of nicotinamide N-methyltransferase and increased formation of 1-methylnicotinamide (MNA) in atherosclerosis. Pharmacol Rep 2009; 61(1):76-85.
64. Sternak M, Khomich TI, Jakubowski A, Szafarz M, Szczepański W, Białas M, Stojak M, Szymura-Oleksiak J, Chłopicki S. Nicotinamide N-methyltransferase (NNMT) and 1-methylnicotinamide (MNA) in experimental hepatitis induced by concanavalin A in the mouse. Pharmacol Rep 2010; 62(3):483-493.
65. Chlopicki S, Kurdziel M, Sternak M, Szafarz M, Szymura-Oleksiak J, Kaminski K, Zoladz JA. Single bout of endurance exercise increases NNMT activity in the liver and MNA concentration in plasma; the role of IL-6. Pharmacol Rep 2012; 64(2):369-376.
66. Fedorowicz A, Mateuszuk Ł, Kopec G, Skórka T, Kutryb-Zając B, Zakrzewska A, Walczak M, Jakubowski A, Łomnicka M, Słomińska E, Chlopicki S. Activation of

the nicotinamide N-methyltransferase (NNMT)-1-methylnicotinamide (MNA) pathway in pulmonary hypertension. Respir Res 2016; 17(1):108.

67. Akalin E, Hendrix RC, Polavarapu RG, Pearson TC, Neylan JF, Larsen CP, Lakkis FG. Gene expression analysis in human renal allograft biopsy samples using high-density oligoarray technology. Transplantation 2001; 72(5):948-953.
68. Allegra A, Marino A, Coffaro F, Lama A, Rizza G, Scaglione P, Sammartano F, Santoro A, Volpes A. Is there a uniform basal endometrial gene expression profile during the implantation window in women who became pregnant in a subsequent ICSI cycle? Hum Reprod 2009; 24(10):2549-2557.
69. Allegra A, Marino A, Peregrin PC, Lama A, Garcia-Segovia A, Forte GI, Nunez-Calonge R, Aqueli C, Mazzola S, Volpes A. Endometrial expression of selected genes in patients achieving pregnancy spontaneously or after ICSI and patients failing at least two ICSI cycles. Reprod Biomed Online 2012; 25(5):481-491.
70. Eyster KM, Hansen KA, Winterton E, Klinkova O, Drappeau D, Mark-Kappeler CJ. Reciprocal communication between endometrial stromal cells and macrophages. Reprod Sci 2010; 17(9):809-822.
71. Debigaré R, Maltais F, Côté CH, Michaud A, Caron MA, Mofarrahi M, Leblanc P, Hussain SN. Profiling of mRNA expression in quadriceps of patients with COPD and muscle wasting. COPD 2008; 5(2):75-84.
72. Kim HC, Mofarrahi M, Vassilakopoulos T, Maltais F, Sigala I, Debigare R, Bellenis I, Hussain SN. Expression and functional significance of nicotinamide N-methyl transferase in skeletal muscles of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2010; 181(8):797-805.
73. Savarimuthu Francis SM, Larsen JE, Pavey SJ, Duhig EE, Clarke BE, Bowman RV, Hayward NK, Fong KM, Yang IA. Genes and gene ontologies common to airflow

obstruction and emphysema in the lungs of patients with COPD. PLoS One 2011; 6(3):e17442.

74. Tanaka Y, Kume S, Araki H, Nakazawa J, Chin-Kanasaki M, Araki S, Nakagawa F, Koya D, Haneda M, Maegawa M, Uzu T. 1-Methylnicotinamide ameliorates lipotoxicity-induced oxidative stress and cell death in kidney proximal tubular cells. Free Radic Biol Med 2015; 89:831-841.
75. Dostalek M, Hardy KD, Milne GL, Morrow JD, Chen C, Gonzalez FJ, Gu J, Ding X, Johnson DA, Johnson JA, Martin MV, Guengerich FP. Development of oxidative stress by cytochrome P450 induction in rodents is selective for barbiturates and related to loss of pyridine nucleotide-dependent protective systems. J Biol Chem 2008; 283(25):17147-17157.
76. Riederer M, Erwa W, Zimmermann R, Frank S, Zechner R. Adipose tissue as a source of nicotinamide N-methyltransferase and homocysteine. Atherosclerosis 2009; 204(2):412-417.
77. Kraus D, Yang Q, Kong D, Banks AS, Zhang L, Rodgers JT, Pirinen E, Pulinilkunnil TC, Gong F, Wang YC, Cen Y, Sauve AA, Asara JM, Peroni OD, Monia BP, Bhanot S, Alhonen L, Puigserver P, Kahn BB. Nicotinamide N-methyltransferase knockdown protects against diet-induced obesity. Nature 2014; 508(7495):258-262.
78. Kannt A, Pfenninger A, Teichert L, Tönjes A, Dietrich A, Schön MR, Klöting N, BlüherM. Association of nicotinamide-N-methyltransferase mRNAexpression in human adipose tissue and the plasma concentration of its product, 1-methylnicotinamide, with insulin resistance. Diabetologia 2015; 58(4):799-808.

79. Bubenek S, Nastase A, Niculescu AM, Baila S, Herlea V, Lazar V, Paslaru L, Botezatu A, Tomescu D, Popescu I, Dima S. Assessment of gene expression profiles in peripheral occlusive arterial disease. Can J Cardiol 2012; 28(6):712-720.
80. Giuliante R, Sartini D, Bacchetti T, Rocchetti R, Klöting I, Polidori C, Ferretti G, Emanuelli M. Potential involvement of nicotinamide N-methyltransferase in the pathogenesis of metabolic syndrome. Metab Syndr Relat Disord 2015; 13(4):165-170.
81. Hong S, Moreno-Navarrete JM, Wei X, Kikukawa Y, Tzameli I, Prasad D, Lee Y, Asara JM, Fernandez-Real JM, Maratos-Flier E, Pissios P. Nicotinamide N-methyltransferase regulates hepatic nutrient metabolism through Sirt1 protein stabilization. Nature Medicine 2015; 21(8):887-894.
82. Parsons RB, Smith ML, Williams AC, Waring RH, Ramsden DB. Expression of nicotinamide N-methyltransferase (E.C. 2.1.1.1) in the Parkinsonian brain. J Neuropathol Exp Neurol 2002; 61(2):111-124.
83. Parsons RB, Smith SW, Waring RH, Williams AC, Ramsden DB. High expression of nicotinamide N-methyltransferase in patients with idiopathic Parkinson's disease. Neurosci Lett 2003; 342(1-2):13-16.
84. Fukushima T, Tawara T, Isobe A, Hojo N, Shiwaku K, Yamane Y. Radical formation site of cerebral complex I and Parkinson's disease. J Neurosci Res 1995; 42(3):385-390.
85. Fukushima T, Kaetsu A, Lim H, Moriyama M. Possible role of 1-methylnicotinamide in the pathogenesis of Parkinson's disease. Exp Toxicol Pathol 2002; 53(6):469-473.
86. Fukushima T, Ohta M, Tanaka K, Kaneko SY, Maeda T, Sasaki A. Niacin metabolism and Parkinson's disease. Asia Pac J Clin Nutr 2004; 13(suppl):176.

87. Williams AC, Cartwright LS, Ramsden DB. Parkinson's disease: the first common neurological disease due to auto-intoxication? QJM 2005; 98(3):215-226.
88. Parson RB, Aravindan S, Kadampeswaran A, Evans EA, Sandhu KK, Levy ER, Thomas MG, Austen BM, Ramsden DB. The expression of nicotinamide N-methyltransferase increases ATP synthesis and protects SH-SY5Y neuroblastoma cells against the toxicity of Complex I inhibitors. Biochem J 2011; 436(1):145-155.
89. Milani ZH, Ramsden DB, Parsons RB. Neuroprotective effects of nicotinamide N-methyltransferase and its metabolite 1-methylnicotinamide. J Biochem Mol Toxicol 2013; 27(9):451-456.
90. Thomas MG, Saldanha M, Mistry RJ, Dexter DT, Ramsden DB, Parsons RB. Nicotinamide N-methyltransferase expression in SH-SY5Y neuroblastoma and N27 mesencephalic neurones induces changes in cell morphology via ephrin-B2 and Akt signalling. Cell Death Dis 2013; 4:e669.
91. Liu KY, Mistry RJ, Aguirre CA, Fasouli ES, Thomas MG, Klamt F, Ramsden DB, Parsons RB. Nicotinamide N-methyltransferase increases complex I activity in SH-SY5Y cells via sirtuin 3. Biochem Biophys Res Commun 2015; 467(3):491-496.
92. Thomas MG, Sartini D, Emanuelli M, van Haren MJ, Martin NI, Mountford DM, Barlow DJ, Klamt F, Ramsden DB, Reza M, Parsons RB. Nicotinamide N-methyltransferase catalyses the N-methylation of the endogenous β-carboline norharman: evidence for a novel detoxification pathway. Biochem J 2016; 473(19):3253-3267.
93. Markert JM, Fuller CM, Gillespie GY, Bubien JK, McLean LA, Hong RL, Lee K, Gullans SR, Mapstone TB, Benos DJ. Differential gene expression profiling in human brain tumors. Physiol Genomics 2001; 5(1):21-33.

94. Yamada K, Miyazaki T, Hara N, Tsuchiya M. Interferon-gamma elevates nicotinamide N-methyltransferase activity and nicotinamide level in human glioma cells. J Nutr Sci Vitaminol (Tokyo) 2010; 56(2):83-86.
95. Xu J, Moatamed F, Caldwell JS, Walker JR, Kraiem Z, Taki K, Brent GA, Hershman JM. Enhanced expression of nicotinamide N-methyltransferase in human papillary thyroid carcinoma cells. J Clin Endocrinol Metab 2003; 88(10):4990-4996.
96. Gehrmann ML, Fenselau C, Hathout Y. Highly altered protein expression profile in the adriamycin resistant MCF-7 cell line. J Proteome Res 2004; 3(3):403-409.
97. Peng H, Yang HW, Song LW, Zhou Z. [Screening the differential expression of adriamycin-resistance related genes of breast cancer by cDNA microarray]. 2009; 89(25):1745-1748.
98. Zhang J, Wang Y, Li G, Yu H, Xie X. Down-Regulation of Nicotinamide N-methyltransferase Induces Apoptosis in Human Breast Cancer Cells via the Mitochondria-Mediated Pathway. PLoS ONE 2014; 9:e89202.
99. Rogers CD, Fukushima N, Sato N, Shi C, Prasad N, Hustinx SR, Matsubayashi H, Canto M, Eshleman JR, Hruban RH, Goggins M. Differentiating Pancreatic Lesions by Microarray and QPCR Analysis of Pancreatic Juice RNAs. Cancer Biol Ther 2006; 5(10):1383-1389.
100. Bi HC, Pan YZ, Qiu JX, Krausz KW, Li F, Johnson CH, Jiang CT, Gonzalez FJ, Yu AM. N-methylnicotinamide and nicotinamide N-methyltransferase are associated with microRNA-1291-altered pancreatic carcinoma cell metabolome and suppressed tumorigenesis. Carcinogenesis. 2014; 35(10):2264-2272.
101. Yu T, Wang Y, Chen P, Li Y, Chen YH, Chen YX, Zeng H, Yu A, Huang M, Bi H. Effects of nicotinamide N-methyltransferase on PANC-1 cells proliferation,

metastatic potential and survival under metabolic stress. Cell Phys Biochem 2015; 35(2):710-721.

102. Xu Y, Liu P, Zheng DH, Wu N, Zhu L, Xing C, Zhu J. Expression profile and prognostic value of NNMT in patients with pancreatic cancer. Oncotarget 2016; 7(15):19975-19981.

103. Roessler M, Rollinger W, Palme S, Hagmann ML, Berndt P, Engel AM, Schneidinger B, Pfeffer M, Andres H, Karl J, Bodenmuller H, Ruschoff J, Henkel T, Rohr G, Rossol S, Rosch W, Langen H, Zolg W, Tacke M. Identification of nicotinamide N-methyltransferase as a novel serum tumor marker for colorectal cancer. Clin Cancer Res 2005; 11(18):6550-6557.

104. Xie X, Yu H, Wang Y, Zhou Y, Li G, Ruan Z, Li F, Wang X, Liu H, Zhang J. Nicotinamide N-methyltransferase enhances the capacity of tumorigenesis associated with the promotion of cell cycle progression in human colorectal cancer cells. Arch Biochem Biophys 2014; 564:52-66.

105. Xie X, Liu H, Wang Y, Zhou Y, Yu H, Li G, Ruan Z, Li F, Wang X, Zhang J. Nicotinamide N-methyltransferase enhances resistance to 5-fluorouracil in colorectal cancer cells through inhibition of the ASK1-p38 MAPK pathway. Oncotarget 2016; doi:10.18632 [Epub ahead of print].

106. Jang JS, Cho HY, Lee YJ, Ha WS, Kim HW. The differential proteome profile of stomach cancer: identification of the biomarker candidates. Oncol Res 2004; 14(10):491-499.

107. Lim BH, Cho BI, Kim YN, Kim JW, Park ST, Lee CW. Overexpression of nicotinamide N-methyltransferase in gastric cancer tissues and its potential post-translational modification. Exp Mol Med 2006; 38(5):455-465.

108. Chen C, Wang X, Huang X, Yong H, Shen J, Tang Q, Zhu J, Ni J, Feng Z. Nicotinamide N-methyltransferase: a potential biomarker for worse prognosis in gastric carcinoma. Am J Cancer Res 2016; 6(3):649-663.

109. Yao M, Tabuchi H, Nagashima Y, Baba M, Nakaigawa N, Ishiguro H, Hamada K, Inayama Y, Kishida T, Hattori K, Yamada-Okabe H, Kubota Y. Gene expression analysis of renal carcinoma: adipose differentiation-related protein as a potential diagnostic and prognostic biomarker for clear-cell renal carcinoma. J Pathol 2005; 205(3):377-387.

110. Sartini D, Muzzonigro G, Milanese G, Pierella F, Rossi V, Emanuelli M. Identification of nicotinamide N-methyltransferase as a novel tumor marker for renal clear cell carcinoma. J Urol 2006; 176(5):2248-2254.

111. Zhang J, Xie XY, Yang SW, Wang J, He CJ. Nicotinamide N-methyltransferase protein expression in renal cell cancer. Zhejiang Univ Sci B 2010; 11(2):136-143.

112. Kim DS, Choi YP, Kang S, Gao MQ, Kim B, Park HR, Choi YD, Lim JB, Na HJ, Kim HK, Nam YP, Moon MH, Yun HR, Lee DH, Park WM, Cho NH. Panel of candidate biomarkers for renal cell carcinoma. J Proteome Res 2010; 9(7):3710-3719.

113. Teng PN, Hood BL, Sun M, Dhir R, Conrads TP. Differential proteomic analysis of renal cell carcinoma tissue interstitial fluid. J Proteome Res 2011; 10(3):1333-1342.

114. Su Kim D, Choi YD, Moon M, Kang S, Lim JB, Kim KM, Park KM, Cho NH. Composite three-marker assay for early detection of kidney cancer. Cancer Epidemiol Biomarkers Prev 2013; 22(3):390-398.

115. Tang SW, Yang TC, Lin WC, Chang WH, Wang CC, Lai MK, Lin JY. Nicotinamide N-methyltransferase induces cellular invasion through activating

matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells. Carcinogenesis 2011; 32(2):138-145.

116. Zhou W, Gui M, Zhu M, Long Z, Huang L, Zhou J, He L, Zhong K. Nicotinamide N-methyltransferase is overexpressed in prostate cancer and correlates with prolonged progression-free and overall survival times. Oncol Lett 2014; 8(3):1175-1180.

117. Kassem HSh, Sangar V, Cowan R, Clarke N, Margison GP. A potential role of heat shock proteins and nicotinamide N-methyl transferase in predicting response to radiation in bladder cancer. Int J Cancer 2002; 101(5):454-460.

118. D'Andrea FP, Safwat A, Kassem M, Gautier L, Overgaard J, Horsman MR. Cancer stem cell overexpression of nicotinamide N-methyltransferase enhances cellular radiation resistance. Radiother Oncol 2011; 99(3):373-378.

119. Pozzi V, Sartini D, Rocchetti R, Santarelli A, Rubini C, Morganti S, Giuliante R, Calabrese S, Di Ruscio G, Orlando F, Provinciali M, Saccucci F, Lo Muzio L, Emanuelli M. Identification and characterization of cancer stem cells from head and neck squamous cell carcinoma cell lines. Cell Physiol Biochem 2015; 36:784-798.

120. Wu Y, Siadaty MS, Berens ME, Hampton GM, Theodorescu D. Overlapping gene expression profiles of cell migration and tumor invasion in human bladder cancer identify metallothionein 1E and nicotinamide N-methyltransferase as novel regulators of cell migration. Oncogene 2008; 27(52):6679-6689.

121. Sartini D, Muzzonigro G, Milanese G, Pozzi V, Vici A, Morganti S, Rossi V, Mazzucchelli R, Montironi R, Emanuelli M. Upregulation of tissue and urinary nicotinamide N-methyltransferase in bladder cancer: potential for the development of a urine-based diagnostic test. Cell Biochem Biophys 2013; 65(3):473-483.

122. Riester M, Taylor JM, Feifer A, Koppie T, Rosenberg JE, Downey RJ, Bochner BH, Michor F. Combination of a novel gene expression signature with a clinical nomogram improves the prediction of survival in high-risk bladder cancer. Clin Cancer Res 2012; 18(5):1323-1333.

123. Tomida M, Mikami I, Takeuchi S, Nishimura H, Akiyama H. Serum levels of nicotinamide N-methyltransferase in patients with lung cancer. J Cancer Res Clin Oncol 2009; 135(9):1223-1229.

124. Sartini D, Morganti S, Guidi E, Rubini C, Zizzi A, Giuliante R, Pozzi V, Emanuelli M. Nicotinamide N-methyltransferase in non-small cell lung cancer: promising results for targeted anti-cancer therapy. Cell Biochem Biophys 2013; 67(3):865-873.

125. Sartini D, Seta R, Pozzi V, Morganti S, Rubini C, Zizzi A, Tomasetti M, Santarelli L, Emanuelli M. Role of nicotinamide N-methyltransferase in non-small cell lung cancer: in vitro effect of shRNA-mediated gene silencing on tumourigenicity. Biol Chem 2015; 396(3):225-234.

126. Kim J, Hong SJ, Lim EK, Yu YS, Kim SW, Roh JH, Do IG, Joh JW, Kim DS. Expression of nicotinamide N-methyltransferase in hepatocellular carcinoma is associated with poor prognosis. J Exp Clin Cancer Res 2009; 16(28):20-29.

127. Mu X, Chen Y, Wang SH, Li M. [The effect of nicotinamide N-methyltransferase overexpression on biological behaviors of SMMC7721 hepatocellar carcinoma cell line]. Sichuan Da Xue Xue Bao Yi Xue Ban. 2013; 44(2):193-195, 217.

128. Sartini D, Santarelli A, Rossi V, Goteri G, Rubini C, Ciavarella D, Lo Muzio L, Emanuelli M. Nicotinamide N-methyltransferase upregulation inversely correlates

with lymph node metastasis in oral squamous cell carcinoma. Mol Med 2007; 13(7-8):415-421.

129. Emanuelli M, Santarelli A, Sartini D, Ciavarella D, Rossi V, Pozzi V, Rubini C, Lo Muzio L. Nicotinamide N-Methyltransferase upregulation correlates with tumour differentiation in oral squamous cell carcinoma. Histol Histopathol 2010; 25(1):15-20.

130. Sartini D, Pozzi V, Renzi E, Morganti S, Rocchetti R, Rubini C, Santarelli A, Lo Muzio L, Emanuelli M. Analysis of tissue and salivary nicotinamide N-methyltransferase in oral squamous cell carcinoma: basis for the development of a noninvasive diagnostic test for early-stage disease. Biol Chem 2012; 393(6):505-511.

131. Pozzi V, Mazzotta M, Lo Muzio L, Sartini D, Santarelli A, Renzi E, Rocchetti R, Tomasetti M, Ciavarella D, Emanuelli M. Inhibiting proliferation in KB cancer cells by RNA interference-mediated knockdown of nicotinamide N-methyltransferase expression. Int J Immunopathol Pharmacol 2011; 24(1):69-77.

132. Pozzi V, Sartini D, Morganti S, Giuliante R, Di Ruscio G, Santarelli A, Rocchetti R, Rubini C, Tomasetti M, Giannatempo G, Orlando F, Provinciali M, Lo Muzio L, Emanuelli M. RNA-Mediated Gene Silencing of Nicotinamide N-Methyltransferase is Associated with Decreased Tumorigenicity in Human Oral Carcinoma Cells. PLos One 2013; 8(8):e71272.

133. Win K.T, Lee SW, Huang HY, Lin LC, Lin CY, Hsing CH, Chen LT, Li CF. Nicotinamide N-methyltransferase overexpression is associated with Akt phosphorylation and indicates worse prognosis in patients with nasopharyngeal carcinoma. Tumour Biol 2013; 34:3923-3931.

134. Ulanovskaya OA, Zuhl AM, Cravatt BF. NNMT promotes epigenetic remodeling in cancer by creating a metabolic methylation sink. Nat Chem Biol 2013; 9(5):300-306.

135. Shlomi T, Rabinowitz JD. Cancer mistunes methylation. Nat Chem Biol 2013; 9(5):293-294.

136. Sperber H, Mathieu J, Wang Y, Ferreccio A, Hesson J, Xu Z, Fischer KA, Devi A, Detraux D, Gu H, Battle SL, Showalter M, Valensisi C, Bielas JH, Ericson NG, Margaretha L, Robitaille AM, Margineantu D, Fiehn O, Hockenbery D, Blau CA, Raftery D, Margolin AA, Hawkins RD, Moon RT, Ware CB, Ruohola-Baker H. The metabolome regulates the epigenetic landscape during naive-to-primed human embryonic stem cell transition. Nat Cell Biol 2015; 17(12):1523-1535.

137. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. METHODS 2001; 25(4):402-408.

138. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227(5259):680-685.

139. Balducci E, Emanuelli M, Raffaelli N, Ruggieri S, Amici A, Magni G, Orsomando G, Polzonetti V, and Natalini P. Assay methods for nicotinamide mononucleotide adenylyltransferase of wide applicability. Anal. Biochem 1995; 228(1):64-68.

140. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72(1-2):248-254.

141. Hassanein M, Callison JC, Callaway-Lane C, Aldrich MC, Grogan EL, Massion PP. The state of molecular biomarkers for the early detection of lung cancer. Cancer Prev Res (Phila). 2012; 5(8): 992-1006.

142. Gibson MK, Forastiere AA. Reassessment of the role of induction chemotherapy for head and neck cancer. Lancet Oncol 2006; 7:565-574.

143. Audrito V, Vaisitti T, Rossi D, Gottardi D, D'Arena G, Laurenti L, Gaidano G, Malavasi F, Deaglio S. Nicotinamide blocks proliferation and induces apoptosis of chronic lymphocytic leukemia cells through activation of the p53/miR-34a/SIRT1 tumor suppressor network. Cancer Res 2011; 71:4473-4483.

144. Ulrey CL, Liu L, Andrews LG, Tollefsbol1 TO. The impact of metabolism on DNA methylation. Hum Mol Genet 2005; 14(1):R139-R147.

145. Bartuś M, Łomnicka M, Kostogrys RB, Kaźmierczak P, Watała C, Słominska E.M., Smoleński R.T., Pisulewski P.M., Adamus J, Gebicki J, Chlopicki S. 1-Methylnicotinamide (MNA) prevents endothelial dysfunction in hypertriglyceridemic and diabetic rats. Pharmacol Rep 2008; 60:127-138.

146. Brzozowski T, Konturek P.C., Chlopicki S, Sliwowski Z, Pawlik M, Ptak-Belowska A, Kwiecien S, Drozdowicz D, Pajdo R, Slonimska E, Konturek S.J., Pawlik W.W. Therapeutic potential of 1-methylnicotinamide against acute gastric lesions induced by stress: role of endogenous prostacyclin and sensory nerves. J Pharmacol Exp Ther 2008; 326:105-116.

147. Sah NK, Seniya C. Survivin splice variants and their diagnostic significance. Tumour Biol 2015; 36:6623-6631.

148. Asanuma K, Moriai R, Yajima T, Yagihashi A, Yamada M, Kobayashi D, Watanabe N. Survivin as a radioresistance factor in pancreatic cancer. Jpn J Cancer Res 2000; 91:1204-1209.

149. Wang L, Zhang GM, Feng ZH. Down-regulation of survivin expression reversed multidrug resistance in adriamycin-resistant HL-60/ADR cell line. Acta Pharmacol Sin 2003; 24:1235-1240.

150. Martinez A, Bellosillo B, Bosch F, Ferrer A, Marce S, Villamor N, Ott G, Montserrat E, Campo E, Colomer D. Nuclear survivin expression in mantle cell lymphoma is associated with cell proliferation and survival. Am J Pathol 2004; 164:501-510.

151. Mahotka C, Krieg T, Krieg A, Wenzel M, Suschek CV, Heydthausen M, Gabbert HE, Gerharz CD. Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas. Int J Cancer 2002; 100:30-36.

152. De Maria S, Pannone G, Bufo P, Santoro A, Serpico R, Metafora S, Rubini C, Pasquali D, Papagerakis SM, Staibano S, De Rosa G, Farina E, Emanuelli M, Santarelli A, Mariggiò MA, Lo Russo L, Lo Muzio L. Survivin gene-expression and splicing isoforms in oral squamous cell carcinoma. J Cancer Res Clin Oncol 2009; 135:107-116.

153. Mahotka C, Wenzel M, Springer E, Gabbert HE, Gerharz CD. Survivin-deltaEx3 and survivin-2B: two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties. Cancer Res 1999; 59:6097–6102.

154. Waligórska-Stachura J, Andrusiewicz M, Sawicka-Gutaj N, Biczysko M, Jankowska A, Kubiczak M, Czarnywojtek A, Wrotkowska E, Ruchała M. Survivin delta Ex3 overexpression in thyroid malignancies. PLoS One 2014; 9:e100534.

155. Nakano J, Huang C, Liu D, Masuya D, Yokomise H, Ueno M, Haba R, Sumitomo S. The clinical significance of splice variants and subcellular localization of survivin in non-small cell lung cancers. Br J Cancer 2008; 98:1109-1117.