Gene expression profiling of Nm23-H2 overexpressing CAL 27 cells using DNA microarray

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Nm23-H1/NDPKA and Nm23-H2/NDPKB belong to a large family of NDP kinases, group of structurally and functionally closely related enzymes. The Nm23/NDPs are known to catalyse the transfer of terminal phosphates from ATP to other NTPs and dNTPs. Besides their role in the maintenance of the cells NTP pool the *nm23* genes/proteins are known to have additional different biological functions, the most important being its metastasis suppressor activity. The complete picture of roles, actions and targets of *nm23* genes/proteins is yet to be discovered. Our goal was to identify the downstream targets of Nm23-H2 by subjecting Nm23-H2 overexpressing CAL 27 cells (oral squamous cell carcinoma of the tongue) to microarray analysis. Using this powerful technology we identified genes, groups of genes and signalling pathways that could be clustered into several groups: apoptosis related genes, cell cycle and DNA damage, *TGFβ* (transforming growth factor beta) signalling pathway and related molecules, *WNT* signalling pathway, differentiation and epithelial structural and related molecules, cell adhesion, metalloproteinases and their inhibitors, vesicular transport related molecules, proteasome associated, ubiquitin mediated proteolysis and several metabolic pathways. Based on these results we suggest that *nm23-H2* might have an important role in oral squamous cell carcinoma which is to be confirmed by future studies.

Key words: nm23-H2, DNA microarray, oral squamous cell carcinoma

Nm23-H1/NDPKA and Nm23-H2/NDPKB belong to a large family of NDP kinases, group of structurally and functionally closely related enzymes [1]. NDPKs are known to catalyze the conversion of dNDPs to dNTPs through a high-energy H118 intermediate [2]. Nm23-H1 and H2 share an 88% amino acid sequence homology, and have been reported to be catalytically active only as homo or heterohexamers. Six other members of the Nm23 family were discovered in humans [3], while all other living beings have at least one *nm23* gene (with the exception of the *Mycoplasma*). The discovery of nm23 in 1988 lounged the metastasis suppressor gene field since it has been identified by Steeg and collaborators upon its reduced expression in melanoma cell lines with high vs. low metastasis potential [4]. Being the first identified metastasis suppressor gene a lot of effort has been put into discovering the mechanisms of its actions in different neoplastic lesions. Almost two decades of investigation resulted in an abundance of different, often contradictory results which prove *nm23* to be involved in tumour formation and progression in different and distinct modalities [5]. Apart from being involved in metastasis formation, the *nm23* gene family has been linked to an impressive number of other basic biological processes: proliferation [6,7], differentiation and development [8-10], cellular trafficking [11], stress and apoptosis [12,13]. These effects probably result from one or more different functions linked to nm23: vesicular trafficking [14,15], microtubule polymerization [16], gene regulation [17] or signal transduction [18]. In addition, it has been proven that, although forming a functional enzyme together, Nm23-H1 and Nm23-H2 have some different, distinct functions. For instance Nm23-H1 provides a source of GAPs for dynamin dependent fission of coated vesicles during endocytosis [14,15], while Nm23-H2 is a transcriptional factor for *myc* oncogene [19,20] and probably participates in β_1 integrin-mediated cell adhesion [21].

Although *nm23* has been intensively studied the exact mechanisms of its actions, the cellular events it controls or participates in or his cellular targets and partners are still to be discovered.

Head and neck squamous cell carcinomas (HNPCC) are the sixth most common malignancy in the world of which oral car-

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cinomas represent a major part. Several research groups published results concerning the role of *nm23* genes in oral squamous cell carcinomas (OSCC) most of which displayed the down regulation of *nm23* in highly invasive carcinomas which would confirm its role as a suppressor gene [22-25]. Our previous results [26], using novel technologies, i.e. DNA microarray technology, confirmed that the overexpression of *nm23-H1* severely changes the expression of an impressive number of genes and groups of genes leading to the conclusion that *nm23-H1* has a major impact in OSCC formation. These results encouraged us to try to identify the state of involvement of *nm23-H2* in the formation of OSCC, identifying its downstream targets. To enlighten the role of *nm23-H2* in OSSC, and point out genes, groups of genes or signalling pathways interacting with Nm23-H2 we have compared gene expression profiles of pEGFPC1-nm23-H2 stably transfected CAL 27 cells (OSSC), with the "empty" vector transfected cells as a control. The results obtained on oligonucleotide microarrays indicate that overexpression of *nm23-H2* might have an impact on tumour progression of OSCC.

Materials and methods

Cells and culture conditions. Human cell line CAL 27 (squamous cell carcinoma of the tongue, poorly differentiated, G3), was obtained by courtesy of Dr. Jeannine Gioanni, Centre Antoine Lacassagne, Nice, France). The cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogene) supplemented with 10% foetal bovine serum (FBS, Invitrogene), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in humidified chamber with 5% CO_2, at 37°C.

Constructs and cloning. nm23-H2 full-length cDNA fragment was subcloned from pcDNA3nm23 constructs (obtained by courtesy of Dr. Marie-Lise Lacombe, Faculte de Medecine Saint Antoine, Paris, France) into pEGFPC1 (Clontech Inc.). Cloning was verified by a set of restriction enzymes, while the existence of fusion GFP-Nm23-H2 proteins was confirmed by Western blotting and fluorescent microscopy.

Stably transfected cell line preparation. For establishing stably transfected clones, CAL 27 cells were seeded on 100 mm Petri dishes and incubated until 80% confluence. The cells were transfected with pEGFPC1 and pEGFPC1-nm23-H2 constructs using Lipofectamine Plus Reagent (Invitrogene) according to manufacturer's instructions. After 24 hours the cells were trypsinised, resuspended in 1:20 ratio in DMEM supplemented with 600 μg/mL geneticin (Sigma), and seeded on six-well plates. The antibiotic supplemented medium was changed every 3-4 days until the development of resistant colonies. The presence of fusion GFP-Nm23 proteins in established clones was verified by fluorescent microscopy and Western blotting (Figure 1 and 2).

Total cellular RNA isolation, microarray hybridization and analysis. Total cellular RNA was isolated from clones expressing EGFP-Nm23-H2 and cells stably transfected with empty construct using TRIzol Reagent (Life Technologies), followed by RNeasy mini spin columns with on-column DNase digestion (Qiagene) according to manufacturer's instructions. The extracted total RNA was electrophoresed through 1% agarose gel to confirm the presence of rRNA and analyze its integrity. The concentration and purity of RNA were determined according to the absorbance measurements at 260 and 280 nm. Additional PCR was performed using primers for intron sequences to exclude possible DNA contamination. For microarray analyses, the concentration of total RNA was adjusted to 2 μg/mL and the standard procedure for preparing the total RNA (15 μg) to be hybridized (first- and second- strand cDNA synthesis, synthesis of biotin-labelled cRNA – in vitro transcription, fragmentation), was followed as recommended by Affymetrix's standard protocol (available on-line, www.Affymetrix.com). Labelled and fragmented cRNA was further hybridized to probes on Affymetrix Human Genome U133A GeneChip (Affymetrix, Santa Clara, CA) containing 22 216 probes, according to manufacturer's instructions. The mRNA expression levels were evaluated using the Affymetrix Microarray Suite 5.0 Software. A comparison analysis was carried out which evaluated the relative change in abundance for each transcript between a base line ("empty" construct containing CAL 27 cells) and experimental sample (CAL 27 clones constitutively expressing EGFP-Nm23-H2).

Real-time PCR. The real-time PCR reactions and analysis were carried out using PTC-200, Peltier Thermal Cycler, (MJ Research) according to the manufacturer's instructions. Total RNA was isolated as described earlier. cDNA was prepared with 2 μg of total RNA in total volume of 100 μL, using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) according to manufacturer's instructions. Real-time PCR reactions were done in 25 μL volume with 2.5 μL cDNA, 0.2 μM of each primer and SYBR Green PCR Master Mix (Applied Biosystems). All PCR reactions were performed according to the following protocol: 95°C for 10 minutes, followed by 40 cycles 95°C for 15 seconds, 62°C for 30 seconds and 60°C for 30 seconds. Every sample was done in triplicate in three independent experiments. The melting curve analysis was performed after each run. The validation experiment was done by making serially diluted cDNA, and results revealed that $2^{-\Delta\Delta Ct}$ method could be used for the calculations (data not shown). Target genes Ct values were normalized against endogenous control 28sRNA. Relative expression was calculated using cDNA isolated from CAL 27 stably transfected with pEGFPC1, "empty", vector as a calibrator.

Primer sequences:
NM23H2/F: 5' CCGGTTGTGGCCATGGTCTG '3
NM23H2/R: 5' CAAGCATCACTCGGCCTGTC '3
TP53/F: 5' TGGAAACTACTTCCTGAAAACAACG '3
TP53/R: 5' ACAGCATCAAATCATCCATTGC '3
CKS2/F: 5' AACACTACGAGTACCGGCATG '3
CKS2/R: 5 'CCACTCCTCTTCAGACATCAG '3

Results

Using the Affymetrix GeneChip HG-U133A oligonucleotide array the expression profiles of CAL 27 cells stably

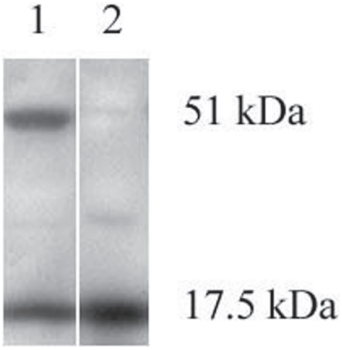

Figure 1.

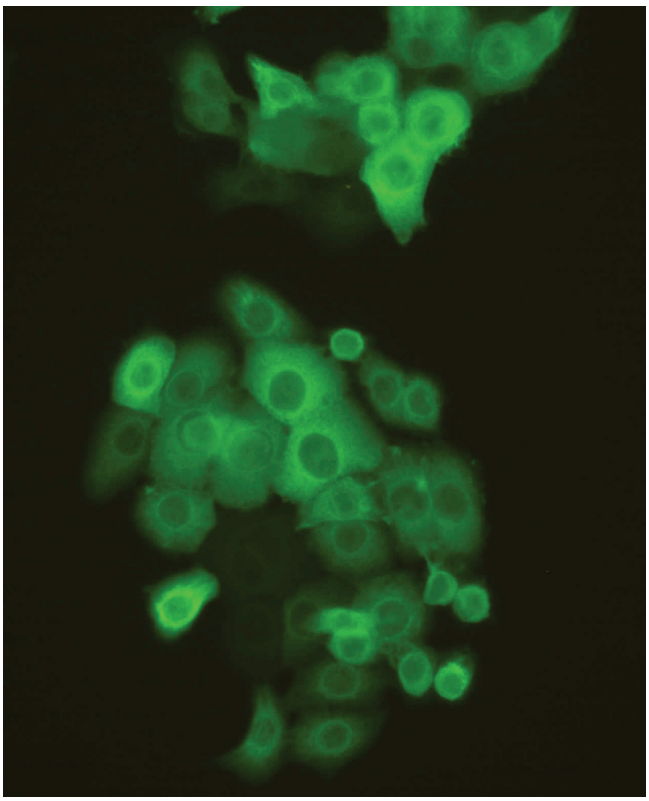

Figure 2.

transfected with pEGFPC1-nm23-H2 were compared to cells transfected with the "empty" construct. The construction of stable clones was verified by Western blot and fluorescent microscopy (figure 1 and 2). Gene annotations were obtained from Affymetrix Netaffx (www.Affymetrix.com) database along with the proposed signalling pathways offered by GenMapp (www.genmapp.org; GenMapp can also bee reached through www.Affymetrix.com). The total of 2380 known and hypothetical genes changed expression levels while 576 of them were altered with the average fold change being more or equal 2 (315 were up-regulated, 261 down-regulated). The filtered genes were roughly clustered into several categories including apoptosis, cell cycle and DNA damage, *TGFβ* signalling pathway and related molecules, *WNT* signalling pathway, differentiation and epithelial structural and related molecules, cell adhesion, *MMPs* (metalloproteinases) and their inhibitors, vesicular transport related molecules, proteasome associated, ubiquitin mediated proteolysis and several others. The differentially expressed gene data list and experiment raw data are deposited at http://www.ncbi.nlm.nih.gov/geo/ (GEO accession no. GSE4069). Table 1 displays the top 12 genes with altered expression, ranked according to their absolute expression change in our experiment. To minimize the effect of the experimental error only the genes with a z-value greater than 2 were taken into account. Analysis was done according to DNA Microarray Data Analysis http://www.csc.fi/oppaat/siru/. Table 2 summarizes the genes and groups of genes with a fold change ≥ 2, and $P \leq 0.01$, which were considered the most important. The *TP53 and CKS2* genes, with an average fold change of 2.6 and 3, respectively, were selected for verification of the microarray results by real-time PCR. The real-time PCR results were proven to be statistically significant according to two-way-ANOVA and Bonferonni post-hoc

Table 1. Genes ranked on the basis of absolute expression change in *nm23-H2* overexpressing CAL 27 cells compared to control

	Gene title	Gene symbol	Change
1	Ankyrin repeat domain 3	ANKRD3	decreased
2	HSPC038, zinc finger protein 706	ZNF706	decreased
3	Polymerase (RNA) II, DNA directed, polypeptide L	POLR2L	decreased
4	Keratin 10	KRT10	decreased
6	H2B histone family, member A	H2BFA	decreased
7	Proteasome subunit alpha type 6	PSMA6	decreased
8	H2B histone family, member S	H2BFS	decreased
9	CDC28 protein kinase regulatory subunit 2	CKS2	decreased
10	ATP-synthase, H+ transporting	ATP5J	decreased
11	Epoxide hydrolase 1, microsomal	EPHX1	decreased
12	Neural precursor cell expressed developmentally downregulated	NEDD8	decreased
11	TP53	TP53	decreased
12	Defender against cell death 1	DAD1	decreased

z-value > 2

Table 2. Selected groups of genes differentially expressed in *nm23-H2* overexpressing CAL 27 clones compared to control

	Gene group	Symbol	Average fold change	Change
A	**Cell-cycle and related molecules**			
	Tumor protein p53	TP53	2.4	decreased
	CDC28 protein kinase regulatory subunit 2	CKS2	2.8	decreased
	WEE1 homolog (*S. pombe*)	WEE1	2.6	decreased
	Cell division cycle 25B	CDC25B	2.0	increased
	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	3.4	increased
	Growth arrest and DNA-damage-inducible, alpha	GADD45A	4.4	increased
B	**Apoptosis**			
	Tumor protein p53	TP53	2.4	decreased
	Protein phosphatase 1, regulatory subunit2	PPP1R2	2.6	decreased
	WEE1 homolog (*S. pombe*)	WEE1	2.6	decreased
	Myeloid leukaemia sequence 1	MCL1	2.4	increased
	Dual specificity phosphatase 1	DUSP1	3.4	increased
	Nuclear factor κ light polypeptide gene enhancer in B-cells inhibitor, α	NFKBIA	3.0	increased
	Tumor necrosis factor receptor superfamily 10b	TNFRSF10B	2.0	increased
	BH3 interacting domain death agonist	BID	2.0	increased
	Tumor necrosis factor receptor superfamily 21	TNFRSF21	2.8	increased
C	**TGFb signalling pathway related molecules**			
	Protein phosphatase 1, regulatory subunit2	PPP1R2	2.6	decreased
	FK506 binding protein 1A, 12 kDa	FKBP1A	2.0	increased
	Dual specificity phosphatase 1	DUSP 1	3.4	increased
	Latent transforming growth factor beta binding protein 2	LTBP2	3.6	increased
	Inhibin β A	INHBA	9.4	increased
D	**Invasion, metastasis and related molecules**			
	Matrix metalloproteinase 1	MMP1	5.2	decreased
	Tissue inhibitor of matrix metalloproteinase 3	TIMP3	2.4	increased
	Matrix metalloproteinase 28	MMP28	3.6	increased
	Catepsin C	CTSC	2.2	increased
	Plasminogen activator urokinase	PLAU	3.0	increased
E	**Integrin-mediated cell adhesion**			
	Integrin α E	ITGAE	2.4	decreased
	Zyxin	ZYX	2.2	increased
	Integrin α 5	ITGA5	2.6	increased
	Integrin α 3	ITGA3	2.2	increased
	Talin 1	TLN1	2.2	increased
G	**Vesicular-mediated transport related**			
	Clathrin, light polypeptide A	CLTA	2.6	increased
	SEC23 homolog B (*S. cerevisiae*)	SEC23B	3.2	increased
	SEC24 related gene family, member D (*S. cerevisiae*)	SEC24D	3.4	increased
	Dynamin	DNM1	2.2	increased
H	**Wnt-signaling**			
	Protein phosphatase 1, regulatory subunit2	PPP1R2	2.6	decreased
	FOS-like antigen 1	FOSL1	4.2	increased
	Plasminogen activator urokinase	PLAU	3.0	increased
	Phospholipase C, beta3	PLCB3	2.0	increased
I	**Epithelial structural and related molecules**		2.8	
	Keratin 1	KRT1	2.8	decreased
	Keratin 10	KRT10	6.2	decreased
	Keratin 13	KRT13	2.0	decreased
	Involucrin	IVL	2.0	decreased
	Keratin 6B	KRT6B	2.4	increased
	Keratin 17	KRT17	2.2	increased
	S100 calcium binding protein A7	S100A7	2.0	increased
	S100 calcium binding protein A8	S100A8	3.0	increased
	S100 calcium binding protein A9	S100A9	4.0	increased
J	**Proteasome associated, ubiquitin mediated proteolysis**			
	Neural precursor cell expressed, developmentally downregulated 8	NEDD8	2.2	decreased
	Proteasome subunit, α type,6	PSMA6	2.0	decreased
	Heat shock 70 kDa protein	HSPA2	4.2	decreased
	DnaJ (Hsp40) homolog, subfamily C, member 15	DNAJC15	2.2	decreased
	Proteasome inhibitor subunit 1	PSMF1	2.0	increased
	Proteasome subunit, β type, 10	PSMB10	2.0	increased
	DnaJ (Hsp40) homolog, subfamily B, member 9	DNAJB9	4.6	increased
	Interferon-stimulated transcription factor 3, gamma 48 kDa	ISFG3G	3.0	increased
	Valosin-containing protein	VCP	2.8	increased
	Major histocompatibility complex, class I, B	HLA-B	2.2	increased
	Major histocompatibility complex, class I, B	HLA-F	2.0	increased

test with $P<0.001$ (Table 3). In addition, the upregulation of *nm23-H2* due to stable transfection was also detected on the microarray, as well as by use of the real-time PCR (Table 3).

Table 3. Validation of DNA microarray results using relative RT-PCR

Target genes	CAL 27 (GFP)	CAL 27	CAL 27/GFP-nm23-H2
nm23-H2	1.000 ± 0.000	1.373 ± 0.163	2.644 ± 0.323
TP53	1.000 ± 0.000	0.679 ± 0.038	0.102 ± 0.017
CKS2	1.000 ± 0.000	1.112 ± 0.128	0.394 ± 0.002

Results are expressed as a fold difference (mean ± SD) in relative expression of target genes, using CAL 27 cells transfected with „empty“ vector as calibrator. Values greater then 2,0-fold or less then 0,5 are considered significant. CAL 27 – untransfected cells, CAL 27 (GFP) – CAL 27 cells transfected with empty vector pEGFPC1, CAL 27 (GFP-nm23-H2) – CAL 27 cells transfected with pEGFP-C1-nm23-H2. According to two-way-ANOVA and Bonferonni post-hoc test the changes in expression, the downregulation of *TP53* and *CKS2* and the upregulation of *nm23-H2* genes, are statistically significant with $P<0{,}001$.

Discussion

In this paper we tried to identify genes, groups of genes and signalling pathways which could be affected by up regulation of *nm23-H2*. We have accomplished this forming a CAL 27 clone (OSCC) stably transfected with pEGFPC1nm23-H2. Subjecting the isolated total RNA from the mentioned clone and control to the DNA microarray we have learned that by overexpressing *nm23-H2* we have changed the expression level of a number of gene groups including: genes related to apoptosis, cell-cycle, *TGFβ*-signalling, *WNT*-signalling, *MMPs* and related molecules, vesicular trafficking and others mentioned with more precision in Table 2. Herein we need to state the limitations of our experiment which was performed once on one stable clone. The performed real-time PCR on three genes (*TP53*, *CKS2* and *nm23-H2*) served as verification of the obtained results. In this paper only genes with the fold change ≥ 2, as well as with a $P < 0.01$ will be discussed.

Our model system displayed several pathways altered by overexpression of *nm23-H2*. The first one is the cell cycle, a complex network of precisely regulated molecules controlling cells life and division. Several key proteins in cell-cycle control have changed their expression level due to overexpression of *nm23-H2* one of them being the cells “lifeguard” *TP53*, down regulated in our model system. It is a protein that has a central role in tumorigenesis (it controls a powerful stress response to DNA damage, hypoxia or inappropriate oncogenic stimulation) [27]. Another important gene/protein with a changed expression rate is the regulatory subunit (*CKS2*) of cyclin dependent kinase 2 (*CDK2*). CDK2 (down regulated in Nm23-H2 overexpressing CAL 27 clones) is important for the transition through the restriction point in the late G1 and it is inhibited by the *p21* (*p21* is up regulated in our model system). It is known that *p21* can be induced by TP53 dependent or TP53 independent pathway. Since *TP53* is down regulated the possible effect of *nm23-H2* overexpression on p21 up regulation could mean the activation of several p53 independent pathways that could lead to several different biological effects (e.g. differentiation) [28,29] that are still to be elucidated. The effect of *nm23* overexpression on growth properties of human oral squamous cell carcinoma were described by Miyazaki et al. [30] where they proved the influence of *nm23-H2* overexpression on proliferation rate of metastatic LMF4 cells, shortening the doubling time from 47 to 28 hours. The effect of expression levels of *nm23* gene on proliferation and cell-cycle control have been reported on other model systems as well, for instance breast carcinoma [6,7,31] where the findings confirmed the involvement on *nm23* genes in breast carcinoma cells, proving that *nm23* silencing leads to lower proliferation rates. Although, as mentioned earlier, the expression rate of a number of important genes/proteins linked to cell-cycle control have been changed, our results on cell-cycle distribution of GFP-nm23-H1 and GFP-nm23-H2 overexpressing CAL 27 clones using flow cytometry (Herak Bosnar et al. Croat Chem Acta 2008, 81, *in press*) displayed no apparent change in the cell-cycle distribution of the mentioned CAL 27 clones compared to control.

Some of the genes mentioned in the group connected to cell-cycle control – TP53, p21, GADD45A – can also be linked to apoptosis which seems to be the most severely altered group of genes in our model system. Table 2 shows nine genes that changed their expression levels due to *nm23-H2* up regulation. Other then those, more than 30 genes in total (*INSIG 1*, *NUP62*, *DLG7*, *IL1B*, *IL8*, *JAG*, *INHBA* etc.) either directly or indirectly linked to apoptosis have changed their expression rate but due to space limitations were not mentioned in table 2 (for details see http://www.ncbi.nlm.nih.gov/geo/, GEO accession no. GSE4069). Recently, there have been several papers that imply the involvement of Nm23 proteins in apoptosis. For instance, the recent findings of Kang et al. suggest that overexpression of *nm23-H2* directly induces apoptosis in connection with Diva, a member of the Bcl-2 gene family in SK-OV3 cells [32]. On the other hand, Fan et al. confirmed that Nm23-H1 (but not Nm23-H2) is activated as a DNase after granzyme A loading or cytotoxic T lymphocyte attack [33].

The changes in expression rate of metalloproteinases (*MMP1* and *MMP28*), one of their inhibitors (*TIMP 3*) together with *catepsin C* and *PLAU* suggest changes in proteolytic activities within the cells constitutively overexpressing *nm23-H2*. Proteases play an important role in invasion and metastases [34]. Metalloproteinases (especially gelatinases - *MMP2* and *MMP9*) are known to be involved in ECM (extracellular matrix) degradation during the invasion process in the carcinomas of the head and neck and can even serve as tumour markers [35]. The *MMP1* is often found to be up regulated in head and neck cancers [36], which our experiments suggest. On the other hand the down regulated *MMP28* is a newly discovered proteinase,

its function in head and neck carcinomas is yet to be revealed. There has been no specific data on the relationship of Nm23 proteins and these particular *MMPs*.

There are several changes in proteins connected to processes engaging integrin-mediated cell adhesion. The complex integrin network plays a key role in tumour invasion. In order to migrate across the extracellular matrix tumour cells have to establish temporary cell contacts with its components [37]. These contacts are mediated by transmembrane heterodimers - *integrins*. The integrin family is a complex one and its expression and function is completely tissue specific [38]. The changed expression of several integrins (*ITGAE*, *ITGA5*, *ITGA3*) together with talin and zyxin suggest major rearrangements in cell adhesion properties. Experiments of Fournier et al. on yeast two-hybrid systems revealed that Nm23-H2 is linked to the cytoplasmic domain of integrin β through the ICAP-1α molecule [21], therefore, confirming the connection of nm23-H2 and the integrin network.

The data obtained on the microarray show that a number of genes involved in endocytosis have also been changed. Recently it has been shown that nm23/NDPK actively contributes to cellular transport supplying GTP for dynamin function [39]. Overexpression of *nm23-H2* stimulated overexpression of dynamin itself (the same feature was observed with overexpression of *nm23-H1*).

In addition, an impressive number of skeletal proteins exhibited severely altered mRNA expression. Five *keratins* (1, 6B, 10, 13, 17), *involucrin* and three members of the *S100 calcium binding proteins*, involved in the development of the cornified envelope changed there expression level [40]. These changes do not came as a surprise because *nm23* genes are well known to participate in differentiation processes in several different organisms although its involvement in the development of stratified oral epithelium has not been described yet.

The overexpression of nm23-H2 also changed the expression of a few members of the WNT-signalling pathway (*PPP1R2*, *FOSL1*, *PLAU*, *PLCB3*). The WNT signalling pathway has long been known to regulate processes connected to growth and patterning during embryonic development and in tissue that undergo constant renewal like intestine and skin [41]. There are several papers indicating the connection between oral carcinoma and the WNT signalling pathway [42]. Until now no connection between *nm23* and the members of WNT-signalling pathway has been observed.

Furthermore, overexpression of *nm23-H2* influenced the expression rate of protease machinery and several heat shock proteins. The association of *nm23* and heat shock proteins was described in the work of Leung and Hightower [43]. Further, our results indicate the involvement of Nm23-H2 in several metabolic pathways (nucleotide metabolism, pyrimidine and purine metabolism, fatty acid metabolism, sugar metabolism, amino-acid degradation etc.). This is to be expected since most of those processes need nucleotides as phosphate donors, and the basic function of Nm23/NDPK is to supply the cell with NTPs and dNTPs.

This is the first article to report on possible targets of Nm23-H2. Recently, we have published results on Nm23-H1 targets obtained on the same model system. Until today, only one group of authors reported data on Nm23 target genes using expression chips [44]. They, however, identified Nm23-H1 downstream targets on the "role model" of Nm23-H1 antimetastatic activity – breast carcinoma cell lines of different metastatic potential. In general, our results obtained on *nm23-H2* microarray resemble those presented by Zhao et al. and Herak Bosnar et al. [26].

Compared to our previous experiments concerning *nm23-H1* overexpressing CAL 27 cells the results obtained with *nm23-H2* overexpressing cells exhibit major similarities when certain groups of genes and pathways are considered. The overexpression of both genes seem to affect the cell cycle, TGFβ signalling, invasion and metastasis related molecules, integrin-mediated cell adhesion, vesicular-mediated transport, proteasome associated, ubiquitin-mediated proteolysis as well as epithelial structural and related molecules. The overexpression of these two related genes do not, however, necessarily affect the same genes but the same groups of genes or related targets that could be involved in the same cell processes. The major difference seems to be the potential involvement of *nm23-H2* in the apoptotic process that couldn't be seen or at least isn't striking in *nm23-H1* overexpressing CAL 27 cells. On the other hand, *nm23-H1* overexpression possibly affects a number of actin binding molecules which isn't true for *nm23-H2* overexpressing cells, at least in our system. The potential similarities and differences do not come as a surprise since the two genes/proteins in some cases perform their cellular function together (NDPK activity, maintenance of the NTP pool) but in other cases have completely distinct cellular roles (transcription factor activities, etc.).

In summary, in this study we have pointed out genes and groups of genes as well as signalling pathways affected by overexpression of one of the NDPK subunits – Nm23-H2/NDPKB in CAL 27 cells. These results suggest the linkage of *nm23-H2* with several biological processes associated with tumorigenesis (cell-cycle and DNA damage, apoptosis) and invasion processes (integrin-mediated adhesion, metalloproteinases and their inhibitors, TGFß signalling and several others). Thus, we conclude, that *nm23-H2* might have an important role in the development of normal oral epithelium as well as in its malignant transformation. Although the role of *nm23* genes/proteins in the development of OSSC is unclear, and the role of these proteins in normal and transformed cell, in general, is yet to be established, this study indicates possible directions of further investigations.

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