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Identification of differentially expressed genes in metastatic and non-metastatic nasopharyngeal carcinoma cells by suppression subtractive hybridization

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Abstract. *Background & objective*: Nasopharyngeal carcinoma (NPC) is an epithelial neoplasm with high occurrence rates in southern China. The disease often metastasizes to regional lymphnodes at a very early stage. Local recurrences and metastasis *occur* frequently in patients with NPC and are a leading cause of death, despite improvements on treatment modalities. The molecular mechanism underlying the metastasis of nasopharyngeal carcinoma remains poorly understood, however, and requires additional elucidation. The aim of this study was to explore possible NPC gene candidates that may play key roles in NPC metastasis. *Methods*: Subtractive suppression hybridization (SSH) was performed to isolate differentially expressed clones between the metastatic 5-8F and non-metastatic 6-10B nasopharyngeal carcinoma cell lines. Differentially expressed clones were screened and confirmed by reverse Northern blotting. The sequences of cDNA fragments were subsequently analyzed and compared to known sequences in Genbank. *Results & discussion*: The SSH library contained thousands of positive clones. Random analysis of 300 clones by PCR demonstrated that 269 clones contained inserted fragments. Reverse Northern blot confirmed that 20 out of 192 clones examined were significantly up-regulated in the 5-8F cell line. Among these 20 clones, 16 were previously identified genes (*flotilin-2*, *ezrin*, *pim-3*, *fli-1*, *mel*, *neugrin*, *znf216*, *ASB1*, *raly, UBE2A*, *keratin6A*, *TMED7*, *EIF3S9*, *FTL*, two ribosomal proteins *RPL21* and *RPL16*), two were predicted genes (*c9orf74* and *MDS006*), and two sequences shared no homology with known genes listed in GenBank and may represent novel genes. The proposed functions of the genes identified in this study include cell signal transduction, cell survival, transcription regulation, cell mobility, protein synthesis, and DNA damage repair. *Flotillin-2*, *fli-1*, *pim-3* and *ezrin* have previously been reported to be associated with tumor metastasis and progression. The remaining up-regulated genes identified in this study have not been reported to be markers of metastasis and may represent new candidates of NPC metastasis-related genes. The results of this study may provide novel points of therapeutic intervention for NPC.

Keywords: Nasopharyngeal carcinoma (NPC), suppression subtractive hybridization (SSH), tumor metastasis, reverse northern blot

1. Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial neoplasm that has a high occurrence rate in several areas of Southern China. The disease is characterized by its capacity to metastasis to regional lymph nodes at a very early stage, and its close tie with Epstein–Barr virus (EBV [31]). Since NPC is highly radiosensitive, radiotherapy remains the principle treatment of choice for this cancer. But local recurrences and metastases are common, and frequently lethal despite improvements in treatment modalities [30]. Unfortunately, the molecular mechanism underlying metastasis of NPC remains poorly understood and requires additional elucidation. In this study we compared the highly metastatic 5-8F and non-metastatic 6-10B NPC cell lines to identify candidate genes related to NPC metastasis.

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Filder and Kripke [6] have proposed that a primary tumor is composed of numerous heterogeneous cell populations, and only a small number of these cells possess the capacity to invade and metastasize. It is shown in multiple studies that metastatic tumor cells have gained properties not possessed by their non-metastatic counterparts. These additional properties are likely reflected by differences in gene expression patterns. Although previous work has shown that latent membrane protein-1 *(LMP-1* [15]), *MMP9* [51], *nm23*, *p16* and *E-cadherin* [19] are associated with metastatic NPC, the molecular mechanism underlying metastasis of NPC remains unknown. Identification of the genes crucial for metastatic dissemination is of great significance, not only for basic understanding of the molecular and cellular processes involved in tumor metastasis, but also for providing potential new therapeutic targets.

The nasopharyngeal carcinoma 5-8F and 6-10B cell lines are ideal for investigation of the quantitative and qualitative changes involved in metastasis. Both cell lines originated from the SUNE-1 NPC cell line and have the same genetic background [40,50], Previous work by Song and others [42] has shown that the metastatic ability of the two celllines in nude mice clearly differed: 5-8F had strong metastatic ability and distant metastasis occurred after four weeks (8/8,100%) while 6-10B had no metastatic ability at all (0/8). The authors also observed that DNA copy number was gained on chromosomes 3p, 7q, 8q, 9q and 10q in the 5-8F cell line, leading them to hypothesize that metastasis-related genes exist in these chromosomal regions [41]. Based on this hypothesis we used the technique of SSH to screen and identify candidate genes that may play important roles in NPC metastasis.

2. Materials and methods

2.1. Cell culture

5-8F and 6-10B cells were kindly provided by Cancer Center, Sun Yet-sen University, Guangzhou, China. The cells were maintained in RPMI 1640 (Invitrogen, USA) supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.2. mRNA isolation and purification

The cells were harvested with 0.25% trypsin and total RNA was extracted with Trizol reagent (Invitrogen, USA). PolyA$^+$ mRNA was purified with Nucleo^{TM} Trap kit (Clontech, Palo Alto, CA) according to manufacturer's instruction.

2.3. Suppression subtractive hybridization

The PCR-selected cDNA subtraction kit was purchased from Clontech (Palo Alto, CA, USA) and used according to the manufacturer's directions. Briefly, polyA$^+$ mRNA was reverse transcribed and the resulting cDNA products digested with *Rsa* I. The digested 5-8F cDNA was then ligated with adapter1 and adapter 2R, respectively. Ligation efficiency was determined by PCR. Two rounds of hybridization were performed using 5-8F as tester and 6-10B as driver. An additional two rounds of subtractive PCR were performed to obtain differential fragments. A detailed description of the protocol can be found in the CLONTECH manual.

2.4. Evaluation of the subtractive efficiency

To evaluate the subtractive efficiency, the relative amount of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) present in the subtracted and un-subtracted cDNA was determined by PCR amplification using the primers provided in the PCR-Select cDNA subtraction kit.

2.5. Construction of subtractive cDNA libraries

E. coli DH5α and pGEM-T easy vector was purchased from Promega (San Diego, CA). The subtracted cDNAs were cloned into a pGEM-T easy vector and the resulting ligation products were then transformed into *E. coli* DH5 competent cells, plated on LB agar plates containing 200 μg/ml ampicillin, 625 μM IPTG and 0.005% X-gal. The presence of cDNA inserts was verified by PCR amplification.

2.6. Screening differentially expressed clones by reverse Northern blotting

Recombinant clones containing cDNA inserts were amplified by PCR using the primers F: 5′-TCGAGCGGGCCGCCCGGGCAGGT-3′ and R: 5′-AGCGGGTGGTCGCGGCCGAGGT-3′ provided in the kit. The resulting PCR products (10 μl) were denatured with 0.4 N NaOH and spotted onto a nylon membrane in duplicate. The membranes were hybridized at 68°C for 16 h with equivalent specific activity of ^{32}P-labeled cDNA probes derived from 5-8F or 6-10B cells, respectively. The membranes were washed in a low stringent solution (2X SSC, 1% SDS; 3 times for 15 min) followed by a high stringent wash

(0.1X SSC, 0.5% SDS; 3 times for 15 min) at 68°C. The membranes were exposed to X-film (Kodak, x-omat BT film) for 48 h at −70°C. The intensity of the hybridization signals was determined with ScanAlyze software provided by Stanford University (http://www.microarrays.org/software.html; elisen@genome.stanford.edu). The analysis performed by ScanAlyze is to estimate the Ch1/Ch2 ratio based on the background corrected mean intensities of the pixels within the spot ellipse [4]. First, the uncorrected mean pixel intensities are exported in the datafile as columns of CH1I and CH2I, along with a count of the number of pixels contained in the spot X). The median value (CH1B, CH2B) intensities of the background pixels also determined. To compute ratios of spot X for use in subsequent analyses, the following formula is used: $\mathrm{Ch1}/\mathrm{Ch2}$ ratio $= (\mathrm{CH1I} - \mathrm{CH1B})/(\mathrm{CH2I} - \mathrm{CH2B})$. Differently expressed clones that were confirmed by reverse Northern blotting were sequenced and analyzed for homology to known sequences in the NCBI database.

3. Results

3.1. Total RNA and mRNA analysis

Integrity of the total and mRNA samples was determined by gel electrophoresis. All samples showed banding patterns typical of high-quality RNA (Fig. 1).

3.2. Rsa I digestion

Both the digested cDNA and undigested cDNA usually appeared as smears. However, their patterns were different for the digested cDNA fragments became shorter after *Rsa* I digestion (Fig. 2).

3.3. Ligation efficiency analysis

The intensity of PCR products amplified using one gene-specific primer (G3PDH 3′ primer) and PCR primer 1 was 25% higher than that of PCR products amplified using two gene-specific primer (G3PDH 3′ primer and 5′ primer), indicating that ligation efficiency was greater than 25% which could ensure the use of tester cDNA in the following hybridization (Fig. 3).

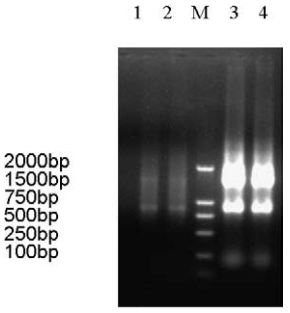

Fig. 1. High integrity and purity of total RNA. Lane 1: mRNA from 5-8F cells; lane 2: mRNA from 6-10B cells; M: DNA marker DL2000; lane 3: total RNA from 5-8F cells; lane 4: total RNA from 6-10B cells.

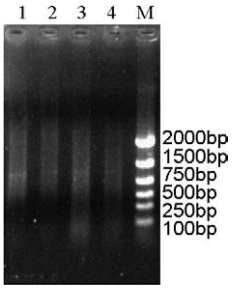

Fig. 2. Analysis of *Rsa* I Digestion. Lanes 1, 2: 5-8F and 6-10B cells dscDNA synthesis before *Rsa*I digestion; lane 3, 4; dscDNA of 5-8F and 6-10B after Rsa I digestion; M: DNA Marker DL2000.

3.4. Analysis of subtraction efficiency

Subtractive efficiency analysis showed (Fig. 4) a reduction in the abundance of non-differentially expressed genes by SSH. G3PDH PCR products could be detected after 18 cycles of amplification in the un-subtracted cDNA library and were saturated after 23–28 cycles. G3PDH was not detected in the subtracted cDNA library until $\geqslant$33 cycles, indicating a dramatic subtraction of commonly expressed genes in these samples.

3.5. Construction of a subtracted cDNA library by SSH

300 clones were randomly picked up from thousands of white clones and plasmids were isolated and amplified by PCR with nest primer 1 and primer 2. As a result, 269 positive clones showed PCR products with a length of 300–1200 bp. Figure 5 showed part of the different lengths of cDNA fragments from white clones amplified by PCR.

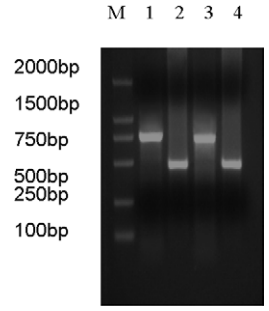

Fig. 3. Results of the ligation efficiency analysis. Lane 1: PCR products using Tester 1-1 (Adaptor1-ligated) as the template and the G3PDH 3′ primer and PCR primer 1. Lane 2: PCR products using Tester1-1 (Adaptor 1-ligated) as the template, and the G3PDH 3′ and 5′ primers. Lane 3: PCR products using Tester1-2 (Adaptor 2R-ligated) as the template, and the G3PDH 3′ primer and PCR primer 1. Lane 4: PCR products using Tester 1-2 (Adaptor 2R-ligated) as the template, and the G3PDH 3′ and 5′ primers. 2% agarose/EtBr gel. Lane M: DNA Marker DL2000.

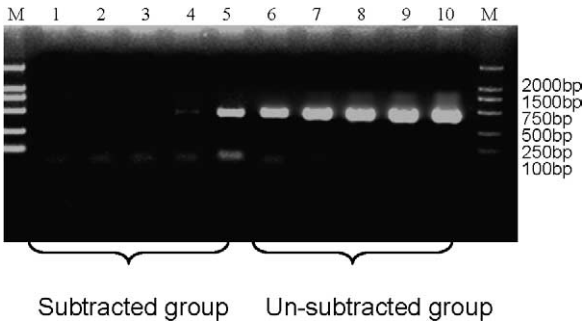

Fig. 4. Reduction of G3PDH abundance by PCR-Select subtraction. Tester cDNA was prepared from 5-8F polyA$^+$ RNA and driver cDNA was prepared from 6-10B polyA$^+$ RNA samples. PCR was performed on subtracted (lanes 1–5) or unsubtracted (lanes 6–10) secondary PCR product with the G3PDH 5′ and 3′ primers included in the kit. Lanes 1 & 6: 18 cycles; lanes 2 & 7: 23 cycles; lanes 3 & 8: 28 cycles; lanes 4 & 9: 33 cycles; lanes 5 & 10: 38 cycles; lane M: DNA marker DL2000.

3.6. Differentially screening of SSH cDNA libraries

A total of 192 clones with inserts were analyzed by reverse Northern blotting to eliminate the false positive clones. The results of hybridization were shown in Fig. 6. The hybridization signals obtained from reverse Northern blot analysis were densitometric scanned of duplicate blots allowed calculation of the signals obtained with cDNA probes derived from 5-8F and 6-10B cells, respectively. Then, following the instruction of ScanAlyze software provide by Stanford University (elisen@genome.stanford.edu) the level of differential expression was represented as a ratio of hybridization signals. The clones with ratio $>$ 2 are regarded as differentially expressed which represents the candidate genes upregulated in 5-8F cells. Of the 192 clones analyzed in the subtractive cDNA library, we have detected 20 clones expressed higher in 5-8F cell.

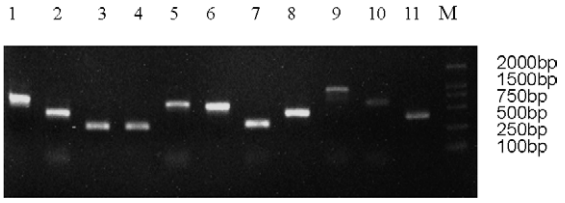

Fig. 5. Different lengths of cDNA fragments from white clones amplified by PCR, lanes 1–11: Randomly-selected white clones, lane M: DNA Marker DL2000.

3.7. Sequencing and homology searching

The twenty clones over expressed in the 5-8F cell line were sequenced for homology to known genes (http://www.ncbi.hlm.nih.gov/BLAST/). BLAST results indicated that 16 out of 20 clones represent known genes, 2 were predicted genes, and two additional cDNAs did not share homology with any known genes and may represent novel genes (Table 1).

4. Discussion

Metastasis and relapse are the main causes of treatment failure leading to NPC patient death. Approximately 70% of patients with newly diagnosed tumors also have detectable lymphnode metastase. Metastasis is a complex process that involves multiple steps, including cell escape from the primary tumor, invasion into the surrounding tissue, and extravasation into the vascular, lymphatic system or peritoneal space, prevention of anoikis (apoptosis induced by anchorage-independence), arrest at a distant site, intravasation into the new environment, evasion of immune responses and proliferation in a new microenvironment. Previous studies have identified many different genes that are involved in metastasis, accelerating or suppressing metastasis by a mechanism of cooperative or inhibitive interactions between genes [49], however, a comprehensive theory on metastisis has not yet been developed.

In this study, we compared the highly metastatic 5-8F NPC cell line with the non-metastasis 6-10B cell line, and searched for differentially expressed genes that might be involved in the process of metastasis.

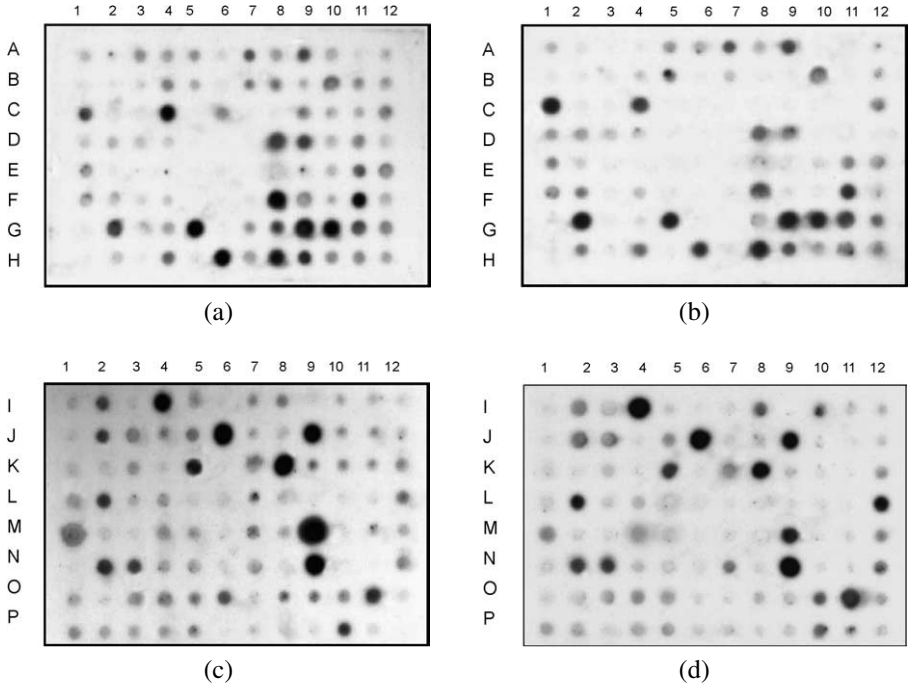

Fig. 6. Screening differentially expressed clones by reverse Northern blotting. cDNA clones randomly picked up from the subtracted library were dotted onto two nylon membranes and hybridized separately with ^{32}P labeled cDNA probes prepared from 5-8F (a,c) cDNA and from 6-10B (b,d) cDNA. Differentially expressed clones were selected for sequence analysis.

Our results showed that the 5-8F cell contained twenty genes over expressed in comparison to the 6-10B cell line.

Flotillin-2(*flot-2*), *ezrin*, *pim-3*, and *fli-1* have previously been reported to play a role in metastasis. Flot-2 is a highly conserved protein isolated from caveolae/lipid raft domains and functions to organize growth factor receptors and modulate downstream signal pathway for cell growth and malignancy. Flot-2 is involved in epidermal cell adhesion [37] and both its mRNA and protein levels are up-regulated in tumorigenic melanoma cell lines *in vitro* [13]. Transfection of full length Flot-2 cDNA constructs into non-metastatic SB2 melanoma cells dramatically alters their metastatic potential in a nude mouse xenograft model. These data demonstrate that over-expression of Flot-2 is sufficient to confer metastatic capacity [13]. Another study has shown that Flot-2 can induce the formation of filopodia and that it appears to play an important role in the initiation of cell invasion [9,14]. Based on these data we speculate that the up-regulation of Flot-2 may contribute to the highly metastatic potential of 5-8F by influencing the NPC cell migration.

Fli-1 (flightless1 homolog) is located on chromosome 11 [16] and is a member of the ETS transcription family. In Ewing Sarcoma, Fli-1 causes transcriptional cofactors to modulate apoptotic pathways and exert antiapoptotic and tumorigenic activities [32]. The over-expression of Fli-1 has been shown to affect cell growth and differentiation of K562 cells [35]. Fli-1 appears to play a pivotal role in the induction and progression of Friend Murine Leukemia Virus (F-MuLV)-induced erythroleukemia. Truong and others [45] demonstrated that Fli-1 binds to the promoter of MDM2 *in vitro* and *in vivo*, leading to MDM2 transcriptional regulation. Fli-1 also has been shown to activate transcription of the Bcl-2 gene and contribute to cell survival [25], as the up-regulation of Bcl-2 results in the evasion of the normal apoptotic program. These studies show that Fli-1 may act as an anti-apoptotic molecule and encourage tumor survival, thus facilitating the process of metastasis.

Our study also indicated that the *Pim-3* gene, a member of the proto-oncogene Pim family, was up-regulated in the 5-8F cell line. Recent work by Fujii and others [8] showed that inhibition of Pim-3 in human hepatoma cells by RNA interference attenuated cell proliferation and apoptosis, suggesting that increased Pim-3 may cause autonomous cell proliferation or prevent apoptosis. The increased expression of Fli-1 and Pim-3 genes in the 5-8F cell line implies that

Table 1
Genes differentially expressed between the highly metastatic and non metastatic nasopharyngeal carcinoma cell lines

Clones	▲Genetic homology	Genebank accession number	Chromosome location	Expression level		Relative ratio (5-8F/6-10B)
				5-8F	6-10B	
G7	*flotillin-2*	M60922	17q11–q12	++	−	>3
K11	*fli-1*	U01184	11q24.1–q24.3	+	−	>3
K10	*ezrin*	NM003379	6q22–q27	+	+/−	>3
C10	*mel*	NM005370	19p13.1–p13.2	+	−	>3
F9	*pim-3*	BC052239	22q13	+++	+/−	>3
B9	*znfF216*	AF062072	9q13–q21	+	+/−	2.34
B7	*asb1*	AF156777	2q37	++	+	2.1
B8	*neugrin*	NM016645	15q26.1	+++	−	>3
B12	*UBE2A*	NM181777	Xq24–q25	+	+	2.07
C6	*keratin6A*	NM005554	12q12–q21	+++	−	>3
I7	*RALY*	AF148457	20q11.21–q11.23	+	−	>3
H7	*FTL*	AY207005	19q13.3–q13.4	++	+/−	>3
D10	*TMED7*	AK074962	5q22.3	+	−	>3
F10	*C9orf74*	AK097029	9q34.13	+	−	2.51
B4	*MDS006*	NM020233	17p13.1	++	–	2.89
C11	*EIF3S9*	U62583	7p22	++	–	>3
G10	*RPL21*	AB007176	13q12.2	++++	+++	2.1
O6	*RPL16*	AB049642	11q12–q13.1	+++	+/−	2.1
A12⋆	*new sequence*	RPL11-501L9	2	+	+/−	2
M9⋆	*new sequence*	RP11-88G17	9	++++++	+++	3.23

▲All identified cDNA sequences are >95% homologous to the known genes in the NCBI Blast search database.
⋆ Represent new sequences have no homology to the known genes in the NCBI Blast search database.
− undetectable; +/− barely detectable; + low expression; ++ moderate expression; +++ moderate to high expression; ++++ or greater, high expression with varying degree of expression.

preventing apoptosis may contribute to the metastatic capacity of this cell line.

Ezrin is a member of the ERM cytoskeleton-associated protein family (Ezrin–Radixin–Moesin). Ezrin serves as an intermediate between the plasma membrane and the actin cytoskeleton [46]. Ezrin has been shown to interact with the cell surface proteins CD44, CD46, CD43, ICAM-1, ICAM-2, and ICAM-3, and also with the proteins actin, tubin, EBP50, E3KARP, MBS, PKA, and RhoGDI. Ezrin is a component of the actin-containing cell surface microvilli, lamellipodia, microspikes and membrane ruffles, structures that have all demonstrated to play an important role in the early steps of tumor invasion [48]. Although the expression of Ezrin is not consistent in different types of tumors, Ezrin is believed to play a role in the metastatic pathway [21]. Up-regulation of Ezrin in malignant esophageal epithelial cells is one of the factors associated with their invasive phenotype [39]. A significant correlation between Ezrin expression levels and poor outcome in pediatric osteosarcoma patients has been reported [22]. In addition, Ezrin is highly expressed in the human pancreatic adenocarcinoma S2-CP9 and S2-VP10 cell lines [27]. In contrast to these data, loss of Ezrin expression in serous ovarian carcinomas was associated with poor survival [26]. These data suggest that Ezrin may play different roles in metastisis depending on tumor origin.

The other genes identified in this study are associated with multiple cellular functions, such as, cell signal transduction, differentiation, transcription regulation, proliferation, and DNA damage repair. Their suggested functions and relation to cancer are illustrated in Table 2. For instance, *EIF3S9(PRT-1*) may separately influence both cell hypertrophy and hyperplasia [10]. Keratin6 has both inflammatory and hyperproliferative properties [12]. Curran and others [2] found an increased level of Keratin6 expression over controls in the epidermis of LMP1 transgenic mice. Overexpression of a subset of rps has been observed in association with cell proliferation. Denis and others [3] reported a correlation between the proliferation rate of cells cultured *in vitro* and the amount of RPL13 mRNA. We hypothesize that RPL21 and RPL16 may have the same

Table 2
Suggested function of the 18 known genes up-regulated in 5-8F cells and their relation to cancer gene predicted by silico cloning method

Gene annotation	Suggested function	Related cancer type	Ref
flotillin-2	Adhension/filopodia formation/ signal transduction	Melanoma	P. Hazarika et al.
fli-1	Anti-apoptosis/signal pathway	Erythroleukemia	A.H. Truong et al.
		Ewing's sarcoma	R. Ramakrishnan et al.
		Giant-cell tumor	K. Scotlandi et al.
ezrin	Migration/adhension/signal pathway/metastasis	Breast cancer	B.E. Elliott et al.
		Osteosarcoma	X. Wan et al.
		Rhabdomyosarcoma	C. Khanna et al.
		Endometrial cancers	Y. Yu et al.
			K. Ohtani et al.
mel	SmallGTP/GDP-binding proteins	Melanoma	A.K. Chakraborty et al.
pim-3	EGFR signal pathway/anti-apoptosis/proliferation	HCC	C. Fujii et al.
ZNF216	Zinc finge protein/signal transduction		J. Huang et al.
asb1	Signal transduction		B.T. Kile et al.
neugrin	Differentiation	Pancreatic ductal adenocarcinomas	S.R. Hustinx et al.
		neuroblastoma	S. Ishigaki et al.
UBE2A	DNA damage repair/cell cycle		P. Kunapuli et al.
			B. Sarcevic et al.
keratin6A	Type II keratin, cytoskeltal		M. Rebecca et al.
RALY	Immune response with EBNA-1/RNA binding protein		G.H. Rhodes et al.
FTL	Metabolism/protein carrier	Neuroblastoma	H.W. Hann et al.
TMED7	Protein transfer		T. Ota et al.
C9orf74*	Unknown hypothetical protein		T. Ota et al.
MDS006*	Unknown hypothetical protein		R.L. Strausberg et al.
EIF3S9(PRT1)	Protein synthesis/cell cycle		P.J. Hanic-Joyce et al.
RPL21	Protein synthesis		J.M. Frigerio et al.
RPL16	Protein synthesis		T. Suzuki et al.

function in proliferation These genes' up-regulation in 5-8F cells may facilitate it survival when it metastasizing to target organs.

In summary, we have identified 20 genes that are differentially expressed between the 5-8F and 6-10B cell lines. Some of these genes have previously been reported to be associated with metastasis in some types of cancer, yet their role in NPC metastasis needs to be further elucidated. The genes Mel and ASB1, and the two novel genes, have not been previously correlated with metastisis of cancer and may represent new candidates. We believe that this work has yield several new targets for further research of the biology of tumor metastosis, as well as possible new targets for NPC treatment.

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