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Identification of a differential expression signature associated with tumorigenesis and metastasis of laryngeal carcinoma

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ABSTRACT

Objectives: Metastasis is the most significant prognostic factor for laryngeal carcinoma which necessitates the identification of molecular alterations associated with metastasis. The identification of such molecular alterations will not only prove useful in treatment but also provide insight into mechanisms of cancer metastasis. The studies conducted so far have not specifically focused on metastasis or invasion pathways. Therefore we investigated the expression profiles with a pathway focused approach.
Materials and methods: Total RNA was extracted from 36 laryngeal tumors and paired cancer free tissue. Expression levels of 88 genes were determined using a PCR array system following cDNA synthesis. Obtained data was used for the calculation of altered expression levels, facilitating relevant algorithms. Significant alterations were determined according to their p-value obtained by Student's *t*-test.
Results: Sixteen genes have shown altered expression when compared with adjacent cancer-free tissue. 2 of these 16 genes have shown differential expression in tumors with neck metastasis in respect to non-metastatic tumors.
Conclusion: We found that TGFB1, TIMP1, c-Myc, SPARC, COL4A2 and SOX4 show altered expression in laryngeal tumors. c-Myc and SOX4 expression is decreased as laryngeal tumors switch to metastatic phenotype.

1. Introduction

Squamous laryngeal cancer is the most common form of head and neck cancers. It has a worldwide incidence of 2.2% and a mortality of 1.2% for both sexes with a 5-year prevalence of 1.5% (Ferlay et al., 2010). Squamous laryngeal cancer is most frequently diagnosed in >60 year old men. Smoking, alcohol consumption and acid reflux are the foremost risk factors for laryngeal cancer (Licitra et al., 2003). Additional culprits include Human papillomavirus (HPV) and genetic susceptibility (Kruszyna et al., 2010; Syrjänen, 2005; Xiao et al., 2013; Ying et al., 2011). HPV infections are known to induce juvenile onset recurrent respiratory papillomatosis which in turn may transform into laryngeal tumors (Syrjänen, 2005). In addition to that recent studies have shown that a positive family history for laryngeal cancer also increases the risk (Garavello et al., 2012; Li et al., 2009). Since the localization of the tumor is the major decisive factor for treatment, tumor invasion has a substantial effect on therapy. Metastasis on the other hand is the most significant prognostic factor. In addition to that, involvement of lymph nodes decreases survival by almost 40% (Mirisola et al., 2011) which necessitates the identification of molecular alterations indicative of invasion and metastasis. The identification of such prognostic markers will not only prove useful in treatment but also serve to reveal the key players of cancer metastasis. Despite that, the studies conducted so far either investigated whole genome or in contrast very few genes, without focusing on metastasis or invasion pathways. Therefore we investigated the expression profiles with a pathway focused approach. We analyzed a set of 88 genes which were previously associated with invasion and metastasis in different cancers. Altered expression profiles were detected by facilitating a qPCR based system and significant changes were identified by statistical analysis. 16 out of 88 investigated genes have shown significantly altered expression in tumor tissue when compared with their cancer free counterpart. 6 most salient of these are *TIMP1*, *MYC*, *SPARC*, *COL4A2*, *SOX4* and *TGFB1*. Two genes, (*c-Myc*, *SOX4*) out of these 16 have been observed to

Abbreviations: RNA, ribonucleic acid; PCR, polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; TIMP1, Tissue Inhibitor of Metalloproteinase I; c-Myc, v-myc avian myelocytomatosis viral oncogene homolog; SPARC, secreted protein acidic and rich in cysteine; COL4A2, collagen alpha-2(IV); SOX4, SRY-related HMG-box-4; TGFB1, Transforming Growth Factor Beta I; HPV, Human papillomavirus; qPCR, *quantitative* polymerase chain reaction; GSEA, gene set enrichment analysis; FDR, false discovery rate; LSCC, laryngeal squamous cell carcinoma.

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dysregulate in metastatic tumors when compared with non-metastatic tumors.

TIMP1 (Tissue Inhibitor of Metalloproteinase I) is a natural inhibitor of matrix metalloproteinases and is located on Xp11.4-p11.23 (Willard et al., 1989). As a result of its function, *TIMP1* directly affects the homeostasis of extra cellular matrix and therefore is linked to carcinogenic processes like tumorigenesis, invasion and metastasis (Cruz-Munoz and Khokha, 2008; Jiang et al., 2002). *c-Myc* (v-myc avian myelocytomatosis viral oncogene homolog) is encoded on 8q24.21. This gene product has a crucial role in carcinogenesis since it not only regulates gene expression by its transcription activity but also effects global gene expression via regulation of histone acetyltransferases (Gearhart et al., 2007). *c-Myc* is activated as a result of external cell stimulus via mitogenic signals. Activation of *c-Myc* has diverse effects on various cellular processes, foremost of which is triggering cell proliferation by upregulating cyclins and downregulating p21 (Dominguez-Sola et al., 2007; Gartel et al., 2001; Taira et al., 1999). The *SPARC* (secreted protein acidic and rich in cysteine) gene, also known as osteonectin, is located on 5q33.1 and codes for a glycoprotein playing a central role in cellular events including differentiation and metastasis by modulating cell-matrix interactions (Brekken et al., 2003; Lane and Sage, 1994). As a result of its de-adhering effect on cells, its role in carcinogenesis and metastasis has been studied in various cancers. *COL4A2* is mapped to 13q33–q34 and codes collagen alpha-2(IV) chain, one of the six subunits of type IV collagen (Boyd et al., 1988). *COL4A2* and *COL4A1*, which were not included in our study, share a common promoter and therefore are usually coexpressed (Kühn, 1995). *SOX4* (SRY-related HMG-box-4), is located on 6pter-p21.3 and encodes a transcription factor functioning as a downstream component of embryonic development and cell fate pathways (Farr et al., 1993). As a transcription factor, it plays a dual role in cell fate determination by inducing the expression of both pro-apoptotic and pro-proliferative genes (Hur et al., 2004; Liu et al., 2006). Overexpression of *SOX4* is shown to be indicative of metastasis in addition to poor prognosis in gastric cancers (Fang et al., 2012). TGFB1, which is mapped to 19q13.1-3, and has a central role in various cellular processes including proliferation and differentiation (Fujii et al., 1986) has been shown to have both positive and negative effects on tumor growth (Derynck et al., 2001).

2. Methods

2.1. Patients and tissue samples

36 patients (male (%): 93.8; female (%): 6.2) with a median age of 57 years were included in our study. Their medical records provided us with necessary medical and demographic data including patient's gender, age and tumor grade and stage. 90.6% of patients were smokers while only 12.5% of them reported alcohol consumption. An informed consent form was obtained from all patients. The study protocol was approved by local ethics committee (Istanbul Faculty of Medicine, November 14 2011, No: 769). Thirty-six tumor and cancer free tissue samples were obtained from previously mentioned patient group by surgical resection. Obtained tissues were immediately frozen in liquid nitrogen and stored until mRNA extraction. All obtained tumor tissues were histologically confirmed. Paired nonmalignant tissues were obtained from more than 10 mm distant surrounding tissue and were histologically confirmed to be free of cancer. In addition to that tumor tissues were categorized in two groups as metastatic and non-metastatic according to their neck metastasis status.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted with High pure RNA isolation kit (Roche Applied Science) from snap frozen tissue samples following tissue homogenization using MagNa Lyser (Roche Applied Science). Extracted RNA samples were stored at −80 °C until analysis and checked for integrity by running on a 1.5% agarose gel (Sigma Aldrich). RNA quantity and purity was determined by absorbance measurement at 260 nm and 280 nm using NanoDrop 2000c UV–Vis Spectrophotometer. 1 μg of total RNA was used for cDNA synthesis with reverse aid first strand synthesis kit (Fermentas Life Sciences) according to the manufacturer's instructions.

Table 1
Genes included in PCR-array.

ADAMTS1	CDH6	CTSL1	HGF	MAP2K4	MMP7	SERPINE1	TIMP4
ALDH3A1	CLDN7	CXCL1	HIF1A	MAP2K5	MYC	SERPINB5	TNC
ANGPT1	COL1A1	CXCL12	HMGB1	MAP2K7	NEDD9	SOX4	TP53
ANGPTL4	COL4A2	CXCR4	ID1	MCAM	NF2	SPARC	VEGFA
CASP8	COL6A1	CXCR6	IGFBP7	MET	NME1	SPP1	ACTB[a]
CCNE2	CSF1	DRG1	IL13RA2	METAP2	NME2	SRC	B2M[a]
CCR7	CSF3	EREG	ISG20	MMP1	NME4	SYK	GAPD[a]
CD44	CST7	FGF8	JAG1	MMP10	PAX5	TFF1	GUSB[a]
CD82	CTGF	FLT1	KISS1	MMP11	PDGFA	TGFB1	HPRT1[a]
CDH1	CTSB	FLT4	KLRC2	MMP13	PLAUR	TIMP1	PGK[a]
CDH11	CTSD	GPI	KYNU	MMP14	PTGS2	TIMP2	PPIA[a]
CDH2	CTSK	GSN	LTBP1	MMP2	RUNX1	TIMP3	RPL13A[a]

[a] Normalizator genes.

2.3. PCR array

All amplification reactions were performed using LightCycler Faststart Sybr Green 1 (Roche Applied Science). Phenix research human tumor invasion panel which is predesigned to contain housekeeping genes for normalization and essential positive and negative controls, was used to determine expression levels of 88 genes for each sample simultaneously. Included genes are listed in Table 1.

2.4. Data and statistical analyses

Data obtained from PCR array was analyzed using a Microsoft Excel spreadsheet with previously described algorithms (Livak and Schmittgen, 2001). Results were then compared and assessed for significance using Student's *t*-test and significant differences in expression levels were denoted as log-transformed ratios to show fold regulation.

Table 2
Differential expression profiles associated with carcinogenic phenotype.

Genes	Tumorigenesis		Metastasis	
	Fold regulation	p-Value	Fold regulation	p-Value
TIMP1	**2.4534**	**0.000002**	−1.391	0.09463
MYC	**4.9295**	**0.000049**	**−1.4925**	**0.033328**
SPARC	**7.541**	**0.000078**	−1.1642	0.618041
COL4A2	**6.7183**	**0.000218**	**−1.5321**	**0.022257**
SOX4	**7.9343**	**0.001993**	−1.1559	0.64802
CTSB	**3.5099**	**0.002436**	−1.2692	0.294364
GPI	**5.1388**	**0.003717**	−1.2811	0.26636
NF2	**4.9867**	**0.004737**	−1.2374	0.37885
MCAM	**7.3518**	**0.004876**	−1.2646	0.305804
GSN	**3.3518**	**0.005007**	−1.0987	0.857857
TGFB1	**16.09**	**0.005529**	1.4006	0.309511
TNC	**9.7232**	**0.006433**	1.0371	0.711601
ISG20	**8.6224**	**0.006749**	1.2383	0.411192
CDH1	**6.6719**	**0.007666**	1.0443	0.694459
MMP11	**9.4355**	**0.008469**	−1.2554	0.329443
TP53	**4.6206**	**0.008822**	−1.3989	0.087358

Significant p-values are given in bold.