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ORIGINAL PAPER

Detection of Deregulated Pathways to Lymphatic Metastasis in Oral Squamous Cell Carcinoma

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Abstract Oral squamous cell carcinoma (OSCC) is a common malignancy, in which lymph node metastasis is a major determinant of outcome. The pathway deregulation resulting from a large number of somatic genetic alterations in the development of the tumor, plays an important role in lymphatic metastasis process. To detect the deregulated pathways to lymphatic metastasis in OSCC, we performed pathway-oriented analysis using gene expression profile from 16 samples without lymphatic metastasis and 27 samples with lymphatic metastasis. We identified seven significantly ($p<0.05$) deregulated pathways: the erythropoietin (EPO) Signaling Pathway, Signaling Pathway from G-Protein Families, Cytokine–cytokine receptor interaction, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, Ribosome, Colorectal cancer, B cell receptor signaling pathway. The biological relevance of these pathways to OSCC is the focus of ongoing studies, as well as complex interactions and crosstalk between them. These pathways might provide additional clues about factors that regulate the course for OSCC patients and might offer new opportunities for therapeutic intervention.

Keywords Deregulated pathway · Lymphatic metastasis · Oral squamous cell carcinomas

Abbreviations

cAMP	cyclic AMP
EPO	erythropoietin
EPOR	erythropoietin receptor
GPCRs	G-protein-coupled receptors
HNSCC	squamous cell carcinomas of the head and neck
JAK	Janus kinase
MAP kinase	mitogen-activated protein kinase
NSCLC	non-small cell lung carcinoma
OSCC	Oral squamous cell carcinoma
STAT	signal transducer and activator of transcription

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common type of carcinoma worldwide, in which lymph node metastasis is a major determinant of outcome [1]. Typically, 50% of patients with OSCC have detectable lymph node involvement at presentation. Only 25% to 40% of patients with lymph node metastasis at presentation survive 5 years, compared to ~90% of patients without metastasis [2]. The mechanisms involved in lymph node metastasis are complex; part of the mechanisms is associated with somatic genetic alterations acquired during the development of the tumor.

Metastasis can be associated with alterations in the myriad cell-signaling pathways that control the crucial events of cell

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function. These pathways are essential to normal cell-growth regulation and cell fate determination, whether that involves programmed cell death (apoptosis) or the capacity to properly differentiate. Genetic aberrations and variations in cellular processes are usually reflected in the expression levels of many genes. Hence, such alterations can potentially be characterized by their gene expression profiles. DNA microarray coupled with bioinformatics tools can detect with remarkable resolution transcriptional signatures [3, 4], and pathway deregulation, which are widely used in attempts to reveal the underlying mechanisms of many diseases at different developmental stages, cellular responses to different conditions, and many other biological phenomena [5].

The aim of this study was to detect the deregulated pathways to lymphatic metastasis in OSCC. We performed pathway-oriented analysis using gene expression profile from 16 samples without lymphatic metastasis and 27 samples with lymphatic metastasis. We identified seven significantly ($p<0.05$) deregulated pathways: the erythropoietin (EPO) Signaling Pathway, Signaling Pathway from G-Protein Families, Cytokine–cytokine receptor interaction, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, Ribosome, Colorectal cancer, and B cell receptor signaling pathway. The biological relevance of these pathways to OSCC is the focus of ongoing studies, as well as complex interaction and crosstalk among them. These pathways might provide additional clues about factors that regulate the course of OSCC patients and might offer new insights into therapeutic intervention.

Materials and Methods

Data Sets: Description, Preprocessing and Normalization

The oral squamous cell carcinomas datasets were obtained from Toruner et al. [6] and O'Donnell et al. [7]. The data were downloaded from http://www.ncbi.nlm.nih.gov/geo/ (Gene Expression Omnibus, accession number: GSE3524, GSE2280) as raw data files (.cel files). Gene expression values were called using the GC-RMA method [8] and data were quantile normalized using the Bioconductor package, affy. The original data contained 22,283 affy probes and 47 samples and covered three groups of samples from OSCC: normal (4 samples), no lymphatic metastasis (16 samples), and lymphatic metastasis (27 samples). In this paper, we compared the two tumor groups (Table 1). If there were multiple probes for the same gene, we reduced the gene probe to gene symbol: for each sample, the expression values of all probes for a given gene were reduced to a single value by taking the maximum expression value. By this process, the 22,283 features were reduced to 13,321 features.

Extraction of Differentially Expressed Genes According to Phenotypes

This step was to screen differential expression according to phenotypes (with or without metastasis). In this study, two genes sets, 49 upregulated genes and 276 downregulated genes (p-value <0.01), were obtained by the application of Data-adaptive method [9] using GEPAS [10].

Detection of Significant Pathways Deregulated to Metastasis

Different repositories of relevant biological pathway information are available and can be used for the pathway analyses of genome-scale experiments, such as KEGG [11] and BioCarta. KEGG is a collection of manually drawn pathway maps on molecular interaction and reaction networks for metabolism, genetic information processing, cellular processes and human diseases (http://www.genome.jp/kegg/kegg2.html), while BioCarta (http://www.bioCata.com) displays gene interactions within pathways for cellular processes, such as apoptosis and cell cycle regulation. In this study, both KEGG and BioCarta were used for only a few pathways in common.

An accumulation of genes within a common pathway which is significantly up- or down-regulated is a clear indication of a possible relationship between this pathway and phenotypes. FatiGo+ [12] were used to test whether the set of up-regulated genes contained significant enrichments on the pathway with respect to the set of down-regulated genes of reference. The programs use a Fisher's exact test for 2×2 contingency tables for comparing two groups of genes and then extracting the pathways whose distribution among the groups is significantly different. A p-value of <0.05 was considered to be statistically significant.

Results

Significant Pathways of Deregulation

Seven deregulated pathways ($p<0.05$) were detected: EPO Signaling Pathway, Signaling Pathway from G-Protein Families, Cytokine–cytokine receptor interaction, JAK-STAT signaling pathway, Ribosome, Colorectal cancer, and B cell receptor signaling pathway. Table 2 shows the detailed p-value and differentially expressed genes for each pathway.

Expression Fold Change of Key Signals in Deregulated Pathways

Deregulation of pathways is directly reflected by expression change of the key signals in the pathways, including

Table 1 Characteristics of the OSCC patients, with TNM staging

Case no.	Sex	Age	Primary site	TNM
GSM42246	M	76	Mandible	T3N2M0
GSM42247	M	76	Mandible	T3N2M0
GSM42248	F	47	Tongue	T2N2M0
GSM42249	F	47	Tongue	T2N2M0
GSM42250	M	42	Mandible	T4aN2M0
GSM42251	M	42	Mandible	T4aN2M0
GSM42252	M	80	FOM/Buccal	T4aN1M0
GSM42253	M	80	FOM/Buccal	T4aN1M0
GSM42254	F	75	Tongue	T2N2M0
GSM42255	F	43	Tongue	T2N2M0
GSM42256	F	47	Tongue	T4aN2bM0
GSM42257	M	80	Gingiva	T4aN2bM0
GSM42258	M	59	Tongue	T3N2M0
GSM42259	M	76	Tongue	T2N1M0
GSM42260	F	75	Tongue	T2N2M0
GSM42261	M	69	Tongue	T1N2M0
GSM42262	M	59	Tongue	T3N0M0
GSM42263	M	83	FOM	T4aN0M0
GSM42264	M	59	FOM	T4aN0M0
GSM42265	M	47	Tongue	T2N0M0
GSM42266	M	48	Tongue	T2N0M0
GSM42267	M	62	FOM/Tonsil	T4aN0M0
GSM42268	M	51	Tongue	T1N0M0
GSM42269	M	63	Tongue	T4N2bM0
GSM42270	M	56	Tongue	T1N2bM0
GSM42271	M	45	Tongue	T3N2bM0
GSM42272	M	50	Tongue	T1N0M0
GSM80460	F	58	FOM	T2N0M0
GSM80461	M	60	FOM	T4N1M0
GSM80463	M	64	Tongue	T1N2bM0
GSM80462	M	72	Vestibule	T2N0M0
GSM80464	M	48	FOM	T4N1M0
GSM80465	M	77	Tongue	T2N0M0
GSM80466	M	66	Tongue	T2N0M0
GSM80467	M	64	Tongue	T4N2bM0
GSM80468	M	32	Tongue	T3N2bM0
GSM80469	M	46	Maxilla	T4N0M0
GSM80470	M	54	FOM	T4N0M0
GSM80471	F	60	FOM	T4N2bM0
GSM80472	M	51	FOM	T2N0M0
GSM80473	F	50	Mandible	T4N0M0
GSM80474	M	47	Tongue	T4N3M0
GSM80475	M	52	FOM	T4N2M0

The cases are indexed by Gene Expression Omnibus (GEO) access number.
TNM Tumor-node-metastasis, *FOM* floor of mouth.

Table 2 Significant pathways of deregulation to lymphatic metastasis in OSCC

Pathways	Genes	*p*-Value	Resource
EPO signaling pathway	EPOR ↑, FOS ↑, JUN ↑	0.041	BioCarta
Signaling pathway from G-protein families	FOS ↑, JUN ↑, GNAS ↑	0.041	BioCarta
JAK-STAT signaling pathway	EPOR ↑, LIF ↑, SOCS3 ↑, CISH ↑ IL22RA1 ↓	0.003	KEGG
Cytokine–cytokine receptor interaction	EPOR↑, INHBB ↑, ACVR2B ↑, LIF↑ IL22RA1 ↓, BMPR1A↓	0.009	KEGG
Ribosome	RPS2 ↑, RPS3 ↑	0.031	KEGG
Colorectal cancer	FOS ↑, JUN ↑	0.031	KEGG
B cell receptor signaling pathway	FOS ↑, JUN ↑	0.031	KEGG

↑ Upregulated, ↓ downregulated *ACVR2B* activin A receptor, type IIB, *BMPR1A* bone morphogenetic protein receptor, type IA, *CISH* cytokine-inducible SH2-containing protein, *EPO* erythropoietin, *EPOR* erythropoietin receptor, *FOS* v-fos FBJ murine osteosarcoma viral oncogene homolog, *GNAS* adenylate cyclase-stimulating G alpha protein, *IL22RA1* interleukin 22 receptor, alpha 1, *INHBB* inhibin, beta B; JUN, jun oncogene, *LIF* leukemia inhibitory factor, *RPS2* ribosomal protein S2, *RPS3* ribosomal protein S3, *SOCS3*, suppressor of cytokine signaling 3

initiative signaling molecules such as receptors and extracellular signaling molecules, mediate signals such as small molecule messengers, and end signals such as transcriptional factors. From this point of view, we grouped the differentially expressed genes for each pathway and calculated the expression fold change (Table 3).

FOS and JUN as Common Endpoints

Our studies found that FOS and JUN were common endpoints shared by the four pathways (Fig. 1), i.e. Colorectal cancer, B cell receptor signaling pathway, EPO Signaling Pathway and Signaling Pathway from G-Protein Families. As the accumulated trigger of upstream signals, FOS and JUN changed their expression most among all key signals in deregulated pathways (Table 3).

Discussion

EPO Signaling Pathway

Erythropoietin (EPO) and erythropoietin receptor (EPOR) are expressed [13] and have a role in promoting invasiveness and metastasis of human head and neck squamous cell carcinoma [14, 15]. The signaling pathway mechanism includes multimerization of the receptor upon ligand binding, activation of MAPK cascade, and phosphorylation and activation of STAT5. For example, EPO, at pharmacological concentrations, can activate JAK2/STAT5 in non-small cell lung carcinoma (NSCLC) cell lines [16].

Signaling Pathway from G-Protein Families

G-protein-coupled receptors (GPCRs) interact with heterotrimeric G proteins to regulate a range of second messenger pathways to enable communication from the cell surface to the nucleus. Gαs-coupled receptors stimulate adenylyl cyclase, which synthesizes cyclic AMP (cAMP) from ATP. Recent investigations have shown that elevation of cAMP resulting from signal transduction from the Gαs protein promotes apoptosis in several cell types including leukemic cells [17], ovarian cancer cells [18], and lymphoma cells [19]. Gαs, encoded by the gene GNAS, activates adenylyl cyclase to produce cAMP. A potential role of Gαs in promoting apoptosis has been proposed through experiments in which increased expression of Gαs has been shown to activate the adenylyl cyclase signal transduction cascade resulting in an accumulation of cAMP [20]. Somatic activating mutations of GNAS principally support a role of GNAS in tumor initiation and/or progression [20–22].

JAK-STAT Signaling Pathway

The JAK-STAT pathway is important for many host responses including defense, differentiation, proliferation, and oncogenesis. It is, therefore, not surprising that numerous regulatory layers exist to modulate this signaling pathway. This includes both negative and positive regulation. The effect of these regulatory processes determines the rate at which STAT signals are transduced. Activation of this pathway is found in squamous cell carcinomas of the head and neck (HNSCC) as a consequence of overexpression of the EGFR prevalent in this cancer type. Here, STAT3 and STAT5 may be the most relevant member of the family [15].

LIF and EPOR over-represented simultaneously accelerated the STAT activity, leading to the up-regulation of CISH and SOCS3 as negative regulator. Therefore, IL22RA1, the receptor of Interleukin-22 which activated the JAK-STAT Pathway, was impeded and STAT1/3 down-regulated, but STAT5 still increased its expression (although not significantly) in our study.

Table 3 Expression fold change of key signals in deregulated pathways

Pathways	Initiative signal						Mediate signal					End signal	
	ACVR2B	BMPR1A	EPOR	IL22RA1	INHBB	LIF	CISH	SOCS3	GNAS	RPS2	RPS3	FOS	JUN
EPO signaling pathway			1.42									2.74	1.68
Signaling pathway from G-protein families									1.29			2.74	1.68
JAK-STAT signaling pathway			1.42	0.75		2.20	1.48	2.51					
Cytokine–cytokine receptor interaction	1.23	0.74	1.42	0.75	1.81	2.20							
Ribosome										1.12	1.37		
Colorectal cancer												2.74	1.68
B cell receptor signaling pathway												2.74	1.68

Cytokine–cytokine Receptor Interaction

Cytokines are small secreted proteins which are important in the regulation of haematopoiesis and immune responses. The regulated physiological processes include the survival and proliferation of haematopoietic progenitor cells, induction of their lineage-specific differentiation, functional activation of mature lymphocytes, the activation and regulation of innate and adaptive immune responses, as well as the selective induction of apoptosis. Cytokines representing only a small number of protein families are crucial in these processes, the regulation of which is important in many areas of disease management.

INHBB-ACVR2B is a ligand-receptor pair of TGF-β family. It is now generally accepted that TGF-β signaling has been shown to promote tumor cell invasiveness and metastasis, but the complete absence of TGF-β signaling in the carcinoma cell population has also been shown. ALK4 binding to ACVR2B (ActR-IIB) in the presence of inhibin ligands, activates downstream SMAD2 and SMAD3 signalling [23], which develops metastatic colorectal cancer and enhances tumor progression [24]. And moreover, BMPR1A, another member of TGF-β family, is expected to be down-regulated, as inactivating this gene can initiate colorectal tumorigenesis [25]. These are consistent with our finding that colorectal cancer pathway was involved in lymphatic metastasis in OSCC.

Stimulation of cell proliferation by cytokines often proceeds via the JAK-STAT pathway in hematopoietic cells and lymphocytes. Both LIF and EPO are IL-6-type cytokines, and both which can activate STAT3 and STAT1, and STAT5 via JAK [26]. LIF stimulates proliferation of rat pituitary tumor cells in culture, and induces tyrosine phosphorylation of STAT3 in the cells [27]. EPO mediates tumor cell invasion, specially in HNSCC cells [28]. Here the upregulation of EPOR and LIF implies that EPO/EPOR and/or LIF mediates invasion and metastasis through JAK-STAT pathway in OSCC. In fact, EPO-mediated activation of JAK2/STAT5 signaling contributes to HNSCC disease progression and metastasis [15].

IL22RA1 contains potential STAT3 recruitment sites and signals through sequential activation of JAK1 and STAT1/STAT3 [29], and its down-regulation may be induced by CISH and SOCS3 increased expression.

Ribosome

The ribosome is a large ribonucleoprotein machine that synthesizes proteins from transcribed mRNA. Several ribosomal proteins have been found to also play roles in induction of apoptosis, suppression of tumors, and regulation of development.

Higher levels of expression of RPS3 in colorectal cancer have been observed, which promotes cell immortalization. RPS2 is high expressed in Human head and neck squamous cell carcinomas [30]. In addition, RPS2 is validated to be novel tyrosine kinase substrates and probably downstream substrates of other growth factor signaling with the exception of EGF [31].

Colorectal Cancer

Accumulating evidence is showing that colorectal cancer is heterogeneous and complex. Relevant to our results, Mutated K-RAS protein activates RAF/MAPK pathway, leading to constitutive growth promotion via FOS and JUN. Another notable subpathway is TGF-β signaling Pathway. Several classes of SMADS, SMADS (SMAD2 and SMAD3)

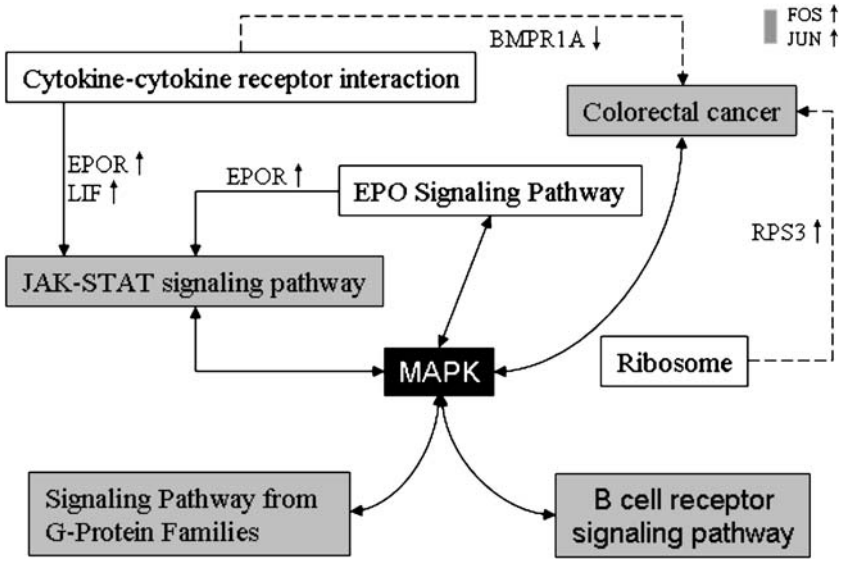

Fig. 1 The interactions of the deregulated pathways to lymphatic metastasis in OSCC. JAK-STAT pathway is the downstream of cytokine–cytokine receptor interaction and EPO Signaling Pathway. MAPK signaling pathway is interacted with five pathways as it is common within them. FOS and JUN are upregulated in four pathways (*gray boxes*). *Dashed lines* represent indirect influence on the pathway

and co-SMAD (SMAD4), play important roles in TGF-β signaling in colorectal cancer cells [32]. As described above, INHBB-ACVR2B interaction or ACVR2B overexpression, the downregulation of BMPR1A, and the overrepresented RPS3 indicates that colorectal cancer pathways are strongly implicated in lymphatic metastasis of OSCC.

B Cell Receptor Signaling Pathway

FOS and JUN, as the direct downstream of activated MAPK pathway and the endpoint of this pathway, increased their expression significantly, which is most likely leading to immuno-response, B cell ontogeny, and allergy. The results of experiments using a mouse model of inflammatory arthritis, showing that inhibition of a transcription factor is sufficient to block generalized symptoms of the disease [33]. It is, therefore, conceivable that FOS and JUN are potential targets for treatment in OSCC.

Interaction of the Pathways

Although the mechanisms and clinical relevance of these seven pathways are described separately, there are multiple interactions between them, reflecting the complexity of OSCC pathogenesis (Fig. 1). The interactions were reflected by up-downstream pathway links and common points/pathway. For example, cytokine–cytokine receptor interaction and EPO Signaling Pathway are the upstream of JAK-STAT pathway. The cascade may promote the invasion and metastasis during the progression of tumor. In reality, EPO mediates invasion by HNSCC cells through JAK-STAT signaling pathway [15]. In addition, although there is no direct evidence, BMPR1A which is a member of cytokine–cytokine receptor interaction initiating colorectal tumorigenesis, may be implicated in colorectal cancer pathway as an upstream signal (Fig. 1).

In the present study, FOS and JUN are common endpoints shared by the four pathways (Fig. 1). As the accumulated trigger of upstream signals, FOS/JUN-regulated gene and their expression may, therefore, be regulated by various oncogenic signaling pathways. This implies FOS and JUN are potential targets for OSCC treatment. MAPK signaling pathway is the direct upstream of FOS /JUN and the common pathway within five of the seven pathways except Ribosome and Cytokine–cytokine receptor interaction, whose activation promotes cell survival, migration and invasion. These demonstrate that one signaling pathway is coordinately associated with another signaling pathway via common factor in the process of carcinogenesis.

The complexity that is associated with lymph node metastasis in OSCC is further convoluted by the disease itself, as many pathways are often misregulated and amplified in cancer cell to promote progression. Detection of deregulated pathways is the first step. Further research is needed to validate their biological relevance and potential of new targets for treatment and drugs.

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