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Genome-wide Expression and Copy Number Analysis Identifies Driver Genes in Gingivobuccal Cancers

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Abstract

The molecular mechanisms contributing to the development and progression of gingivobuccal complex (GBC) cancers—a sub-site of oral cancer, comprising the buccal mucosa, the gingivobuccal sulcus, the lower gingival region and the retromolar trigone—remain poorly understood. Identifying the GBC cancer-related gene expression signature and the driver genes residing on the altered chromosomal regions is critical for understanding the molecular basis of its pathogenesis. Genome-wide expression profiling of 27 GBC cancers with known chromosomal alterations was performed to reveal differentially expressed genes. Putative driver genes were identified by integrating copy number and gene expression data. A total of 315 genes were found differentially expressed ($P \leq 0.05$, $\log_{2}FC > 2.0$) of which eleven genes were validated by real-time quantitative reverse transcriptase-PCR (qRT-PCR) in tumors (n=57) and normal GBC tissues (n=18). Overexpression of *LY6K*, in chromosome band 8q24.3, was validated by immunohistochemical (IHC) analysis. We found that 78.5% (2,417/3,079) of the genes located in regions of recurrent chromosomal alterations show copy number dependent expression indicating that copy number alteration has a direct effect on global gene expression. The integrative analysis revealed *BIRC3* in 11q22.2 as a candidate driver gene associated with poor clinical outcome. Our study identified previously unreported differentially expressed genes in a homogeneous subtype of oral cancer and the candidate driver genes that may contribute to the development and progression of the disease.

INTRODUCTION

Copy number alterations (CNAs) of the genome have a strong effect on gene expression and determine cancer cell survival and progression (Hyman et al., 2002; Albertson et al., 2003; Järvinen et al., 2006; Järvinen et al., 2008). CNAs observed in tumors often involve a large group of genes located consecutively along the same chromosome. Only a minority of these

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genes are likely to be the true “driver” genes contributing to tumorigenesis. The other genes, called “passenger” genes, may be altered simply because of their chromosomal location and proximity to the target gene(s) (Leary et al., 2008). Driver genes activate the neoplastic process and mutations in these genes contribute to the transformation of normal cells to proliferating cancer cells (Akavia et al., 2010). Distinguishing driver genes from passenger genes is an intriguing question in cancer genetics. One approach to answer this question is to integrate copy number, gene expression and clinical data to distinguish passenger genes, of which the expression mainly reflects the genomic copy number, from driver genes, which promote the disease and have clinical relevance as biomarkers (Santarius et al., 2010).

This study presents a gene expression analysis of oral cancer, focusing on squamous cell carcinomas of the “gingivobuccal complex” (GBC). The GBC is composed of the buccal mucosa, the gingivobuccal sulcus, the lower gingiva and the retromolar trigone. It is the most common sub-site of oral cancer, closely linked to the habit of chewing betel quid containing tobacco in the Indian population (Pathak et al., 2005). Oral cancers from different sites within the entire oral cavity differ in their biological and clinical behavior, leading to discordance in genomic and transcriptomic profiling studies (Estilo et al., 2009). Most reports on gene expression profiling in oral cancer have either used tumors from multiple, heterogeneous sites in the oral cavity (Roepman et al., 2006; Chen et al., 2008; Sticht et al., 2008; Méndez et al., 2009) or have focused on tongue cancers (Carinci et al., 2005; Shimada et al., 2005; Zhou et al., 2006; Ye et al., 2008; Estilo et al., 2009). There are no previous studies on gene expression profiling of GBC cancers.

Our array comparative genome hybridization (aCGH) study revealed clinically relevant CNAs in oral cancers (Ambatipudi et al., 2011), but the relative importance of individual genes in these regions remained elusive. To our knowledge, only a single study has utilized the potential of integrating copy number and gene expression data for identifying genes associated with poor survival using tumor cells from metastatic lymph nodes in heterogeneous oral sites (Xu et al., 2010). Therefore, we performed an integrative analysis of primary tumors from a homogeneous site to understand the influence of CNAs on the gene expression signature in GBC cancer.

MATERIALS AND METHODS

Tissue Specimen Collection and Tumor Microdissection

This project was approved by the Institutional Review Board and the Local Ethics Committee of Tata Memorial Hospital (Approval number HEC No.559 of 2008). Written informed consents were obtained from all study participants. Neo-primary oral tumor samples were collected from 57 patients undergoing surgery at the Head and Neck Unit and from the ICMR National Tumour Tissue Repository, Tata Memorial Centre, Mumbai. Patients received neither radiation nor chemotherapy before surgery. Tissues with more than 70% tumor content were subjected to RNA extraction. A possible presence of Human Papilloma Virus (HPV) DNA in tumor specimens was analyzed by polymerase chain reaction (PCR) using GP5+/GP6+ (L1 region) generic primers (Ambatipudi et al., 2011). Non-inflamed GBC mucosa was collected from 18 healthy individuals to serve as reference tissues. All the samples were snap frozen in liquid nitrogen and later kept at -80°C until they were used. For the microarray gene expression analysis, a test set consisting of 27 GBC cancers (with known copy number alterations) and five normal GBC samples (4 individual normal and 1 pool of RNA from 9 other normal samples) was used. The differentially expressed genes were validated by real-time quantitative reverse transcriptase-PCR (qRT-PCR) on an independent set of GBC samples from 30 patients and 9 additional normal subjects. Immunohistochemical (IHC) analysis was performed on 55 formalin fixed paraffin-embedded tumors and 12 unrelated GBC tissues.

RNA Isolation and Gene Expression Analysis

RNA was isolated from the tumor and normal tissues using the RNeasy mini kit according to the manufacturer's instructions (Qiagen GmbH, Hilden), as described in the Supporting Information Text. The Whole Human Genome Microarray Kit, 4×44K (Agilent Technologies, Santa Clara, CA) with 43,376 oligonucleotide reporters was used for the gene expression study. Single color labeling, hybridization and data extraction were done according to the manufacturer's protocol. Briefly, 1.65 μg of RNA was labeled with Cy3 dye using the Quick Amp one color labeling kit (Agilent Technologies, Santa Clara, CA). Slides were washed, dried and scanned on an Agilent high resolution scanner (Agilent Technologies, Santa Clara, CA) with default parameters followed by feature extraction using the FE software version 10.5 (Agilent Technologies). All data generated was MIAME compliant and the raw data is deposited in the GEO database (accession number GSE23558) following the instructions on the MGED Society website <http://www.mged.org/Workgroups/MIAME/miame.html>.

Data Pre-processing

The Agilent 44K array has multiple oligonucleotide reporters for some genes and many reporters that cannot be unambiguously interpreted as representing the expression level of a single gene (Gertz et al., 2009). Hence, in this study, we restricted the analysis to 25,505 reporters annotated "fully valid". Raw intensity values were first normalized and quality filtered with the Agi 4×44 PreProcess Bioconductor package (Gentleman et al., 2004). After initial quality filtering, 15,946 reporters mapping to 11,718 genes were used for the subsequent expression analysis.

Analysis of Gene Expression Data

We identified differentially expressed genes with fully valid reporters using the Bioconductor package limma (Smyth, 2004). Each reporter was tested for a difference in the mean logarithmic intensity between the tumor and the normal samples by a moderated t-test. When there was more than one fully valid reporter for a gene, p-values for the significance of the differential expression were combined using the method of R. A. Fisher (Elston, 1991). Gene-specific p-values were then corrected for testing across multiple genes by the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). A corrected p-value below 0.05 was considered statistically significant. The genes so obtained were considered differentially expressed if the absolute logarithmic expression fold-change to the base 2 (logFC) between the groups was above 2, corresponding to a 4-fold expression change.

Integrative Gene Expression and Copy Number Analysis

For 93 recurrent copy number alterations (Ambatipudi et al., 2011), the set of genes covered by each CNA was determined. Then, for each CNA, the expression Y_i of reporter i was modeled according to the linear model $E[Y_i] = X\alpha_i$, where $\alpha_i = (\alpha_i^N, \alpha_i^{OC}, \alpha_i^{CNA})$ describes the average expression values for normal, tumors, and tumors with a CNA at the locus of reporter i , respectively. Ambiguous genes with spatially overlapping CNAs of opposing type were excluded. Tests on the averages were done by means of the moderated t-test and combined p-values for genes were then computed from the p-values of reporters with Fisher's method, as described above. To test the overall gene dosage effect of a given CNA and each of 14 peak regions of maximal recurrence (Ambatipudi et al., 2011) within a CNA, we first computed the logFC value for each tumor sample and compared it with the averaged value in normal samples of all valid reporters of the genes located in the CNA under consideration. Then we tested with a single-sided t-test if the logFCs in cases with the alteration were, on average, different from those without the alteration. The tail of the t-test was chosen to test for a higher fold-change for gains, and a lower fold-change for losses.

Survival Analysis

The survival analysis was based on the Cox proportional hazard model. The risk was modeled by the function $\exp(-\lambda_0(t)\beta^T X)$, where $\lambda_0(t)$ denotes the baseline risk, β represents a vector of risk coefficients and X denotes the expression of genes. The coefficients β were estimated from the data. Additional backward model selection was performed based on the Bayesian Information Criterion (BIC) to avoid overfitting. The R package *survfit* was used for these analyses.

Validation of Copy Number Alteration of the 8q24.3 Locus by Interphase Fluorescence In Situ Hybridization (I-FISH)

The copy number status of the 8q24.3 locus was evaluated by dual color I-FISH. The BAC clones RP11-642A1 (8q24.3) and RP11-73M19 (8 centromere), used for FISH, were obtained from the Children's Hospital Oakland Research Institute (CHORI) BACPAC resources center. A centromeric probe served as hybridization control and was used as a reference to determine the copy number status of the 8q24.3 locus. FISH images were captured under a fluorescence microscope (Axioskop II, Carl Zeiss, Germany) and analyzed using the ISIS imaging software (Metasystems, Altlußheim, Germany).

Literature Mining

A literature search was done to find genes that were previously suggested to have either differential expression or copy number alterations in oral cancer using PubMed, PubMedCentral, and the Science Citation Index and was completed in May 2010. We noted whether the changes in copy number or expression were associated with prognosis. Although there were numerous reviews of genomics and oral cancer, we could not find a comprehensive list of implicated genes in any review.

Real-time qRT-PCR Analysis of Differentially Expressed Genes

The qRT-PCR assays were carried out for selected candidate genes found significantly upregulated (*SPPI*, *CA9*, *HOXC9*, *TNFRSF12A*, *LY6K*, *INHBA*, *FST*, *MFAP5* and *DHRS2*) or downregulated (*MAL* and *GPX3*). Two genes (*TSN* and *SLC4A1AP*), which showed no significant differential expression, were used to validate the findings of gene expression data. The fluorescent TaqMan probes were obtained from Applied Biosystems. The assay IDs and the protocol details are provided in Supporting Information Table 1 and Supporting Information Text, respectively.

Statistical Analysis of qRT-PCR Data

The Shapiro-Wilk test was used to assess the normal distribution of differentially expressed genes in normal and tumor samples. A p-value below 0.05 was considered statistically significant. The genes that followed a normal distribution were compared using an independent samples t-test, while a Mann-Whitney test was used for comparison of the genes which did not follow a normal distribution. For eleven selected genes, the Pearson correlation between logFC values of the microarray and qRT-PCR was computed. The analysis was done in SPSS 15.0.

Immunohistochemical Analysis

The immunohistochemical analysis of formalin fixed paraffin embedded tissues was performed using the Vectastain[®] universal elite ABC kit (PK-6200, Vector Laboratories, Inc, Burlingame, CA). Tissue sections of 5 μ m thickness were deparaffinised, rehydrated and treated with peroxidase block. After heat based antigen retrieval, sections were incubated with normal horse serum. The sections were incubated overnight with rabbit polyclonal anti-human LY6K antibody (HPA017770, Prestige Antibodies, Sigma-Aldrich

St. Louis, MO) at a dilution of 1:250. These sections were next kept for 30min with biotinylated universal secondary antibody solution followed by incubation with Vectastain® elite ABC reagent for 30min. The immunoreaction in tissue sections was visualized using 3, 3'-Diaminobenzidine tetrahydrochloridehydrate (D5637, Sigma-Aldrich St. Louis, MO). The slides were finally counterstained with haematoxylin. Sections treated with normal rabbit serum were used as negative controls.

RESULTS

Patient Characteristics

The clinicopathological and demographic characteristics of all tumor samples are summarized in Table 1. The patients in this study cohort were predominantly male tobacco habitués. Tumors were of moderate or poor grade, mainly of pTNM stages III or IV and approximately half the number of cases showed lymph node invasion. Thirty additional GBC cancers with similar characteristics and nine unrelated normal samples from GBC sites were used for validating the findings by qRT-PCR. All samples were unrelated to human papillomavirus infection (Ambatipudi et al., 2011). Case-wise details for all GBC cancer patient samples used for gene expression, qRT-PCR and IHC analysis are summarized in Supporting Information Table 2.

Differentially Expressed Genes in GBC Cancers

We found 315 genes differentially expressed (80 upregulated and 235 downregulated) in GBC cancers, as shown in Supporting Information Table 3. The 40 genes with the strongest fold-change in either direction are listed in Supporting Information Table 4.

Validation of Gene Expression Results by qRT-PCR

From the 80 genes with the most extreme logFC values (40 upregulated and 40 downregulated), we selected 11 genes for individual validation either due to the absence of prior reports (see literature search below) or their potential as putative serum biomarkers. Real-time qRT-PCR was done for the genes *SPPI*, *CA9*, *HOXC9*, *TNFRSF12A*, *LY6K*, *INHBA*, *FST*, *MFAP5*, *DHRS2*, *MAL*, and *GPX3* in both the test and the validation sets, to confirm the results obtained by genome-wide differential gene expression analysis in the test set only. We found statistically significant differences in the ΔC_T values between normal and tumor specimens. Overexpressed genes showed lower ΔC_T values in tumors as compared to normal tissue while the opposite was observed for underexpressed genes. *TSN* and *SLC4A1AP* showed no significant change in the ΔC_T values (Figure 1A) validating the gene expression findings. The correlation of the microarray and the qRT-PCR data for the test set was determined for all validated differentially expressed genes. Our qRT-PCR results correlated strongly with those from microarray analyses ($R^2=0.92$, $P<0.0001$; Pearson correlation test) (Figure 1B). Real-time qRT-PCR confirmed the direction of the change in the validation set of tumors ($n=30$) and in normal samples ($n=9$).

Integrative Analysis of Gene Expression and Copy Number Alterations

We found that 26.3% (3,079/11,718) of the analyzed genes were located in regions of recurrent CNAs found in a previous study (Ambatipudi et al., 2011), as shown in Supporting Information Table 5. Of these genes, 78.5% (2,417/3,079) showed copy number dependent expression effects, meaning higher average expression in tumors with a copy number gain, or lower average expression in tumors with genomic losses. The frequency of a copy number dosage effect on gene expression did not differ significantly between gains (1,751/2,203 genes with increased expression in gains) and losses (673/890 with decreased expression in losses).

The recurrent CNAs showing a high concordance (mean $|\log_{2}FC| > 0.5$, $P < 0.05$) with gene expression results were the losses of 7q31.1, 9p21.3, 11q22.3-q23.1, 11q23.3-q25 and 22q11.23 as well as the gains of 9p21.3-p24.3, 11q22.1-q22.2, and 20p11.21 (Figure 2 and Supporting Information Table 6). The amplification of 11q22.1-q22.2 spanning genes *MMP7*, *TMEM123*, *BIRC2*, *YAP1*, *BIRC3* and *MMP10* showed the highest mean fold-change in copy number dependent overexpression ($\log_{2}FC = 1.71$). Conversely, the most extreme underexpression ($\log_{2}FC = -0.91$) was observed in the region of 9p21 containing *CDKN2B*, *CDKN2A*, and *MTAP*.

Survival Analysis

A univariate survival analysis of differentially expressed genes revealed no significant association with survival after correction for multiple testing. Multivariate Cox proportional hazards models were used to assess the contribution of genes residing in focal CNAs that contained fewer than ten genes with valid reporters and were significantly correlated with expression ($P < 0.05$; t-test). These restrictions were made to keep the model complexity tractable and to preserve power otherwise lost due to multiple testing. The best subsets of genes for prediction, which may also contain no genes, were selected by the BIC.

For the losses of 5q35.3 and 7q31.1, the BIC method did not select any significant genes. For the deletion of 9p21.3, a combination of *CDKN2A* ($\lambda = 0.287$, risk coefficient) and *CDKN2B* ($\lambda = -0.472$) was selected ($P = 0.07$, likelihood ratio test). For one of two focal gains on 11p11.2, the BIC selected the *ARFGAP2* ($\lambda = 1.62$) and *PACSN3* ($\lambda = -1.53$) genes ($P = 0.054$). For the gain of 11q13.2-q13.3, the best subset consists of the genes *FADD* ($\lambda = 2.47$), *FGF3* ($\lambda = -1.84$), *ORAOVI* ($\lambda = 1.9$), and *PPFIA1* ($\lambda = -2.74$; $P = 0.006$). For the gain of 11q22.1-q22.2, the covariates in the Cox model could be reduced to only *BIRC3* ($\lambda = 0.65$; $P = 0.04$). Kaplan-Meier plots with risk terciles for all multivariate cases are shown in Figure 3A–C. A Kaplan-Meier plot demonstrating association of high *BIRC3* expression with poor survival is shown in Figure 3D. Finally, we computed the best combination of genetic predictors from the combined set of genes selected for each CNA. BIC model selection yielded the combination of *ARFGAP2* ($\lambda = 3.51$), *BIRC3* ($\lambda = 0.97$), *ORAOVI* ($\lambda = 2.90$), and *PPFIA1* ($\lambda = -1.32$), as the optimal multivariate predictor ($P = 0.002$, Figure 3E).

FISH Analysis for Validation of 8q24.3 Gain

Of all genes on chromosomal region 8q24, *LY6K* showed the highest overexpression ($\log_{2}FC = 2.3$) irrespective of the copy number change which was apparent from the homogeneous overexpression in the samples with or without 8q24 gain ($\log_{2}FC = 0.54$, $P = 0.19$). The copy number increase of the 8q24.3 locus was validated using interphase FISH (Supporting Information Figure 1).

IHC Analysis of LY6K

The immunohistochemical analysis showed higher protein abundance at the plasma membrane and cytoplasm in cancer specimens than in normal tissues (Figure 4), thereby validating the findings of the expression analysis.

Literature Search

The literature search identified 277 genes previously suggested to have either copy number variations or differential expression in oral cancer. The full list is shown as Supporting Information Table 7 and is based on 73 references. Of these 277 genes, 214 genes that have both a valid reporter on the microarray and an adequate signal in the experiments are marked with a "#". Only 39/214 genes (Supporting Information Table 8A) were mentioned in

multiple papers, even though we explicitly searched for replication papers, and 28/214 genes (Supporting Information Table 8B) had evidence for both copy number changes and differential expression. Among these 28 genes, 23 genes had evidence for gain and overexpression, 4 had evidence for loss and underexpression, and the gene *FAT1* had conflicting evidence on expression. There were 129 genes with evidence of only differential expression, and 57 genes had evidence only of copy number changes (Supporting Information Tables 8C and 8D). Surprisingly, these 57 genes are divided into 24 genes with gains and 33 genes with losses, significantly different from the gain/loss pattern for the 28 genes with both types of evidence ($P < 0.0005$, two-sided Fisher's exact test).

DISCUSSION

We performed gene expression profiling of 27 GBC tumors to delineate differentially expressed genes and did an integrative analysis to identify candidate driver genes. Three hundred and fifteen genes were found differentially expressed in GBC cancers and selected genes were validated by qRT-PCR and IHC on an independent set of tumors and unrelated normal GBC tissues. The integrative copy number and gene expression analysis revealed a large number of genes in which the two types of changes were associated. Our study identified novel genes with a potential role in tumorigenesis and demonstrated the utility of an integrative approach in identifying candidate driver genes.

To our knowledge, this is the first study that provides a comprehensive genome-wide gene expression profile and integrative analysis of GBC cancers. A recent integrative study identified genes associated with poor survival in metastatic OSCC (Xu et al., 2010), but the use of microdissection in isolating tumor cells from nodes did not account for tumor-stromal interactions at the primary site which play an important role in the development and progression of OSCC (Chen et al., 2008). Hence, we used primary GBC tissues and avoided microdissecting tumor cells. Field cancerization has been reported to alter the genetic profile of histologically normal tissues surrounding primary tumors (Braakhuis et al., 2003). To avoid any such influence, unrelated, non-inflamed normal tissues were accrued from the GBC site.

A number of genes found differentially expressed in our study cohort have been previously reported in oral or head and neck cancers including *INHBA*, *SERPINE1*, *GBP5*, *MMP10*, *MMP3*, *PTHLH*, *KRT4*, and *MAL* (Chung et al., 2006; Chen et al., 2008; Ye et al., 2008; Méndez et al., 2009). In addition, several previously unreported genes such as *LY6K*, *MFAP5*, *HOXC9*, *TNFRSF12A*, *DHRS2*, *FST*, and *GPX3* were also found to be differentially expressed and warrant further investigation. A majority of these differentially expressed genes (*MMPs*, *PTHLH*, *INHBA*, *LY6K*, *MFAP5*) are secretory proteins and may be utilized as potential serum biomarkers to assess disease aggressiveness.

The literature search revealed 214 genes with valid reporters in our study that had been previously implicated in oral cancer. Among these, 42 genes showed differential expression in our study (Supporting Information Table 8E); for all but one gene, the direction of expression change agreed with previous reports. Forty-one of the 42 genes were among the 129 genes for which the prior evidence was only about differential expression; only one gene, *FSTL3*, had prior evidence for both differential expression and copy number alterations. Interestingly, 32 of the 42 genes with prior evidence were previously reported in at least one of two studies (Nagata et al., 2003; Kornberg et al., 2005), while other studies in our literature search had few if any genes replicated in our GBC cohort. The concentration of the replicated prior evidence in two studies suggests that the failure to replicate genes across multiple studies of oral cancer may be in part due to heterogeneity between tumor sites which was avoided in our study.

The gain of chromosome band 8q24 is a frequent event in head and neck squamous cell carcinomas, including oral cancers (Huang et al., 2002; Pathare et al., 2009). Previous reports have suggested the overexpression of the known oncogene *MYC* as the underlying event in the gain of 8q24 (Bhattacharya et al., 2009; Grisanzio and Freedman, 2010). We did not find overexpression of *MYC*, within this region, indicating that *MYC* is not primarily regulated via copy number gain; a similar negative finding about *MYC* has been reported in cervical cancers (Lando et al., 2009). From our study, it appears that *LY6K* is constitutively overexpressed either by copy number changes, or by yet unknown mechanisms. *LY6K* belongs to the LY6 family and by sequence analysis, is predicted to encode a protein anchored to the cell membrane via glycosyl-phosphatidyl-inositol (GPI). *LY6K* is also predicted to be secreted and involved in cell signaling because these functions have been proven for other members of the LY6 protein family (de Nooij-van Dalen et al., 2003), although the precise role of *LY6K* in carcinogenesis is unknown. Ishikawa et al. have suggested the use of *LY6K* as a potential serologic and histochemical biomarker for lung and esophageal cancers and its potential activation in a range of tumor samples, including cervical cancers (Ishikawa et al., 2007). We, for the first time, show the overexpression of *LY6K* in GBC cancers, which adds to the tumor types possibly benefiting from drugs targeting *LY6K*.

The mystery of how *LY6K* could be both GPI-anchored and secreted was resolved in a recent study of the orthologous mouse *Ly6k* in which it is shown that there are two protein isoforms, one anchored and one secreted (Maruyama et al., 2010). The two *Ly6k* isoforms differ by a post-translational modification that changes the molecular weight, and hence they cannot be distinguished by RT-PCR or any other technique that considers the cDNA/mRNA (Maruyama et al., 2010).

MFAP5 (*MAGP2*) located in 12p13.1-p12.3 is one of the genes showing over-expression independently of copy number status in GBC cancers that has not been previously reported as differentially expressed in oral cancer. *MFAP5* is a secreted factor and an important component in the assembly of microfibrils. It has recently been shown to mediate ovarian tumor cell survival and endothelial cell motility via the alpha(V)beta(3) integrin receptor, and it was associated with chemoresistance and poor clinical outcome (Penner et al., 2002; Mok et al., 2009). The novel finding of increased expression of *MFAP5* may be due to specific relevance of this gene to the GBC site or to preponderance (in our cohort) of locally advanced lesions that show higher tendency to migrate and attract blood vessel formation. Considering the secretory nature of *MFAP5* and its role in cell motility and survival, we hypothesize that it may be utilized as a serum biomarker to assess disease aggressiveness.

An analysis of affected signaling pathways using the DAVID database (Supporting Information Text and Supporting Information Table 9) indicated an overrepresentation of differentially expressed genes in the KEGG pathways related to extracellular matrix receptor interaction, cytokine-cytokine receptor interaction, focal adhesion, arachidonic acid metabolism, chemokine signalling, cell adhesion molecules and complement and coagulation cascades. Yet, the power of this approach is challenged by the large number of tests performed, and the significance is weak after correcting for multiple testing. We selected a few genes from these pathways based on their very high (*SPP1*, *CA9*, *FST*, *INHBA*, *TNFRSF12A*) or low (*GPX3*) expression levels in tumors. We also validated genes previously unexplored in oral cancers, including *LY6K*, *HOXC9*, *DHRS2* and *MFAP5*. We selected *MAL* because it was reported to be associated with disease progression and metastasis in head and neck tumors (Beder et al., 2009), which may share some risk factors with GBC tumors.

Cytokine-cytokine receptor interactions in the tumor milieu play an important role in cancer pathogenesis. Cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis (Dranoff, 2004). Our analysis revealed overexpression of *INHBA*, *TNFRSF12A* and *CXCL* chemokines (*CXCL10*, *CXCL12*, *CXCL13*). One function of cytokines is to trigger inflammatory responses that play decisive roles at different stages of tumor development (Grivennikov et al., 2010). Arachidonic acid and its inflammation-related metabolites play a role in tumor biology (Hyde and Missailidis, 2009). We report the downregulation of many genes in this pathway including *ALOX12*, *EPHX2*, *PTGDS* and *GPX3*. Reactive oxygen species (ROS) generated during tobacco usage are reported to cause increased genomic instability, growth and invasion (Nair et al., 2004; Yu et al., 2007). As most of the patients in our study are tobacco users, we speculate that the reduced levels of the *GPX3* enzyme may make the oral epithelial cells unable to quench ROS, contributing to tumor progression.

Integration of gene expression data with array CGH data revealed that gene expression and copy number alterations were coherently linked for the majority of genes located in recurring CNAs. Because the pattern of genomic alterations varies substantially between patients, the correlation between expression and copy number implies a similar heterogeneity among patients, at the level of gene expression. As a consequence, it is a challenge to identify subsets of patients with differential prognosis or who respond to different treatments based on either genomic alterations or gene expression alone. Another challenge is the identification of candidate driver genes and their distinction from passengers (Akavia et al., 2010). Based on the wide-spread gene-dosage effect on mRNA expression observed here, it appears that a correlation of gene expression and DNA copy number is, on its own, a rather unspecific criterion. On the contrary, one may argue that driver genes essential for carcinogenesis should be constitutively differentially expressed also in the absence of a CNA, and may be distinguished from passenger genes whose expression mostly reflects the copy number status. An additional criterion to identify a driver gene could be the correlation of expression with clinicopathological parameters such as lymph node invasion or survival (Santarius et al., 2010).

To get further insights into the role of genes located in regions of recurring genomic alterations, we assessed their prognostic relevance. As the identification of genes underlying large CNAs remains difficult, we restricted the analysis to genes in focal alterations that were found to correlate with copy number status. We found that the expression of genes located in 2 distinct regions, 11q13–2 q13.3 and 11q22.1–q22.2 serve as the strongest survival predictors.

A gain of the 11q13.2–q13.3 region containing 12 genes was observed in 37.7% of all the GBC cancers analyzed. Our results identified the expression of *ORAOVI*, *FGF3*, *FADD*, and *PPFIA1* as strong predictors of patient survival. A systematic analysis of gene expression and amplification of the same region by Huang et al. (2006) found that, among others, the overexpression of *ORAOVI*, *FADD*, *PPFIA1* was copy number dependent, while the overexpression of *ANO1* (Anoctamin 1, formerly *ORAOV2*, oral cancer overexpressed 2) was not (Huang et al., 2006). These findings are in agreement with a recent report by Ayoub et al., where a copy number dependent expression of *PPFIA1*, *ORAOVI*, *FADD*, and *ANO1* was observed. Further, the study identified *ANO1* and *ORAOVI* as candidate genes for predicting metastasis in head and neck squamous cell carcinoma (Ayoub et al., 2010).

The consistent overexpression of *ORAOVI* (oral cancer overexpressed 1) in all three studies, including ours, underlines its functional importance in head and neck, and oral squamous cell carcinomas. *ORAOVI* regulates angiogenesis and tumor growth in OSCC (Jiang et al., 2008) and a recent report identified its role in regulating cell cycle and apoptosis (Jiang et

al., 2010). Moreover, we found a tendency for overexpression of *ANO1* in tumors ($\log_{2}FC = 0.7$, $P = 0.2$) in addition to a strong copy number dependent effect ($\log_{2}FC = 1.61$, $P = 10^{-5}$), in line with recent reports (Huang et al., 2006; Kashyap et al., 2009; Ayoub et al., 2010). In our study, however, *ANO1* was not found as a survival predictor. *FADD* (Fas (TNFRSF6)-associated via death domain) mediates apoptotic signaling by binding to different cell-surface receptors of the tumor necrosis factor superfamily. Recently, *FADD* was found to be overexpressed in another study of oral cancer (Prapinjumrune et al., 2010). *FGF3* (fibroblast growth factor 3) expression was found to be variable in this study, in agreement with the findings of Huang et al. (2006), and a lower expression was associated with worse prognosis. *PPFIA1* (protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 1) regulates cell-matrix interactions and displayed a copy number dependent overexpression ($\log_{2}FC = 1.29$, $P = 3 \cdot 10^{-6}$). Interestingly, higher *PPFIA1* expression was found to coincide with a better prognosis, in line with recent findings in HNSCC (Tan et al., 2008). It thus appears that the complex process of tumor progression is a consequence of the combined effect of multiple driver genes in 11q13.

We previously reported a gain of 11q22.1-q22.2 in 14.8% of the GBC cases and found it to be associated with poor prognosis in oral cancers (Ambatipudi et al., 2011). This amplicon caused the highest average fold-change in gene expression and it includes the genes *BIRC3*, *BIRC2*, *TMEM123*, *YAP1*, *MMP7*, and *MMP10* (Supporting Information Table 6). The expression of the first five increases approximately by a factor of two in the presence of the CNA. *MMP10*, however, is consistently overexpressed ($\log_{2}FC=5.1$, $P=0.002$), irrespective of the copy number status. Snijders et al. reported the importance of this amplicon in oral cancers suggesting that *MMP7*, *YAP1*, *BIRC2*, and *BIRC3* could be driver genes (Snijders et al., 2005). However, they observed very little overexpression of *BIRC3* (baculoviral IAP repeat-containing 3) when amplified (Snijders et al., 2005). Overexpression of *BIRC2* and *BIRC3* which encode IAP1 and IAP2 respectively, is consistent with other evidence that these apoptosis-related genes are coordinately regulated (Conze et al., 2005). Overexpression of *BIRC3*, but not *BIRC2*, was associated with poor clinical outcome in our study cohort thus suggesting that *BIRC3* is the driver gene.

BIRC3 encodes a protein (IAP2) that inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2. Tumors with 11q22.1-q22.2 amplification could thus gain aggressiveness through copy number dependent *BIRC3* overexpression which helps tumor cells to evade hypoxia induced apoptosis (Kilic et al., 2007; Lando et al., 2009). Overexpression of *BIRC3*/cIAP2 has been implicated in the progression of both solid and haematological malignancies (Friboulet et al., 2008; Karasawa et al., 2009; Grzybowska-Izydorczyk et al., 2010; Wu et al., 2010). When cellular inhibitor of apoptosis proteins (c-IAPs) are overexpressed or overly active, as in many cancers, cells are no longer able to die in a physiologically programmed fashion and become increasingly resistant to standard radiation and chemotherapies (Hunter et al., 2007). Higher levels of cIAP2 inhibit the effectiveness of commonly used cancer drugs 5-Fluorouracil (Karasawa et al., 2009) and cisplatin (Wu et al., 2010). Both cIAP1 and cIAP2, and also other proteins with IAP domains can be inhibited pharmacologically and are the molecular targets of various cancer clinical trials (Vucic and Fairbrother, 2007).

Cancer is a complex multigenic and multifactorial disease (Hanahan and Weinberg, 2011) and the presence of a single driver gene on a chromosomal location may not be the sole requirement for GBC cancer progression. It is important to identify the gene(s) in a region of copy number change that provide a selective advantage during the process of clonal evolution and tumor progression. Based on our integrative analysis, we hypothesize that the driver genes *ORAOV1* and *BIRC3* may interact with each other or with other genes in their

11q amplicons to evade apoptosis in GBC cancer cells. The possible interactions between genes on 11q and the underlying molecular mechanisms need further investigation.

In summary, our study analyzed the gene expression patterns in GBC cancers. We reported several novel and previously reported genes contributing to various pathways deregulated in GBC cancers. The integrative analysis revealed a widespread dosage effect of CNAs on global gene expression. The study could identify the driver gene *BIRC3* amongst several individually expressed genes located in the focal gain of 11q22.1-q22.2 previously shown associated with poor clinical outcome in oral cancers. Further, the genes so identified, including some of the secretory factors we validated, may serve as potential biomarkers with prognostic value to assess clinical outcome of GBC cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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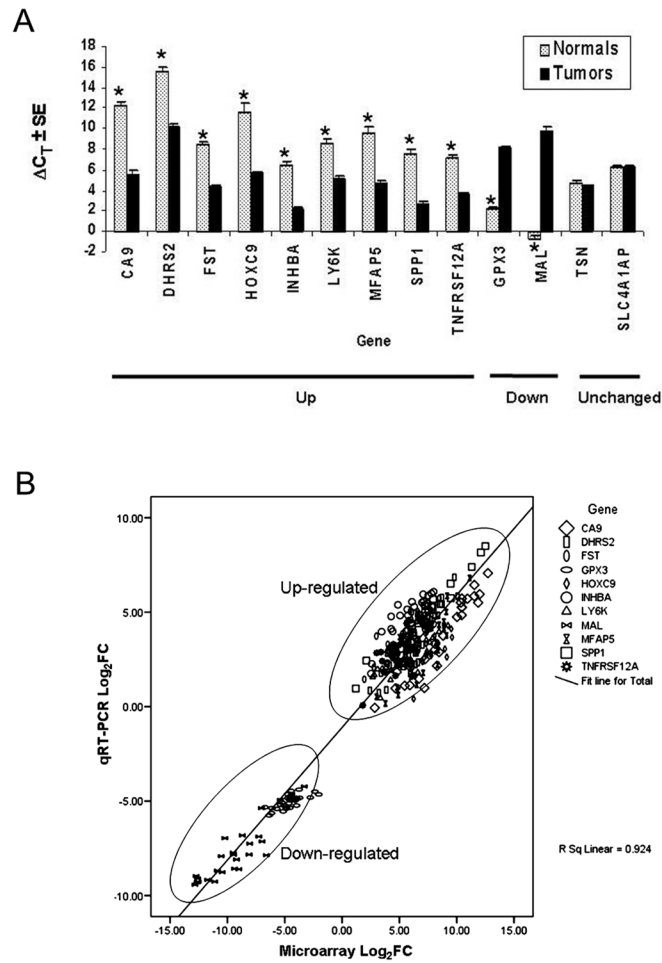
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**Figure 1.**

A: Validation of eleven genes found differentially expressed in the test set by qRT-PCR (tumors $n = 57$ and Normals $n = 14$). Each tumor-normal pair of vertical bars represents a gene grouped according to the type of differential expression. The height of a bar is the average difference in PCR cycles compared to 18S RNA, ΔC_T . Error bars represent the standard error. On the right, *TSN* and *SLC4A1AP* represent genes without any change in expression between tumors and normals. Statistically significant differences (see Methods in Supporting Information Text for details) in the ΔC_T -values are indicated by stars (*).

B: Correlation analysis between the microarray and qRT-PCR fold-change values for eleven differentially expressed genes in test set of 27 GBC samples. Plot Shows the Log_2FC values for microarray data on the x-axis versus Log_2FC values for qRT-PCR data on the y-axis.

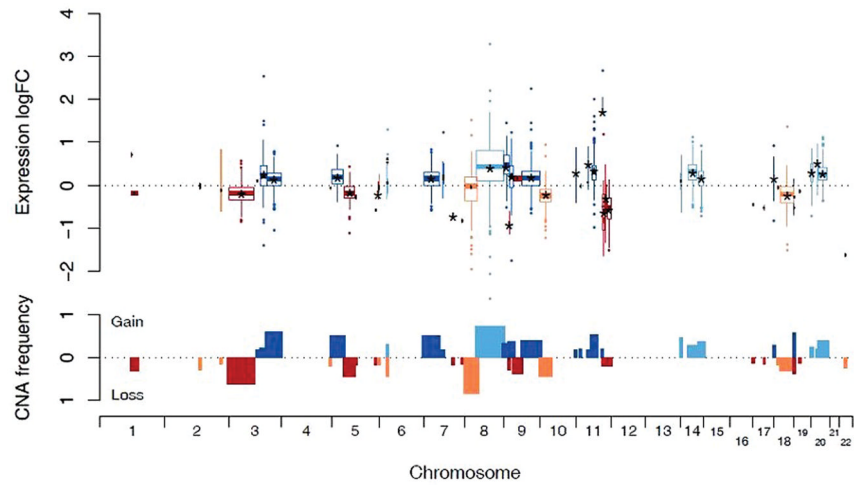


Figure 2.

Effects of gene dosage on the underlying genes in GBC tumors ($n = 27$). In the upper panel, each of the 93 recurring CNAs is represented by a boxplot describing the distribution of the logarithmic expression fold-change of all genes located in the CNA compared to those cancer cases without the aberration. The position on the x-axis and the width of each box denotes the genomic position and spatial extent of the alteration respectively. Blue colors represent gains and show a positive expression fold-change while red color representing losses show a negative fold-change. Any significant average fold-changes ($P < 0.05$, Benjamini-Hochberg adjusted t-test) are indicated by star marks (*). In the lower panel, bars indicate the observed frequencies of CNAs; gains are plotted upwards and losses downwards.

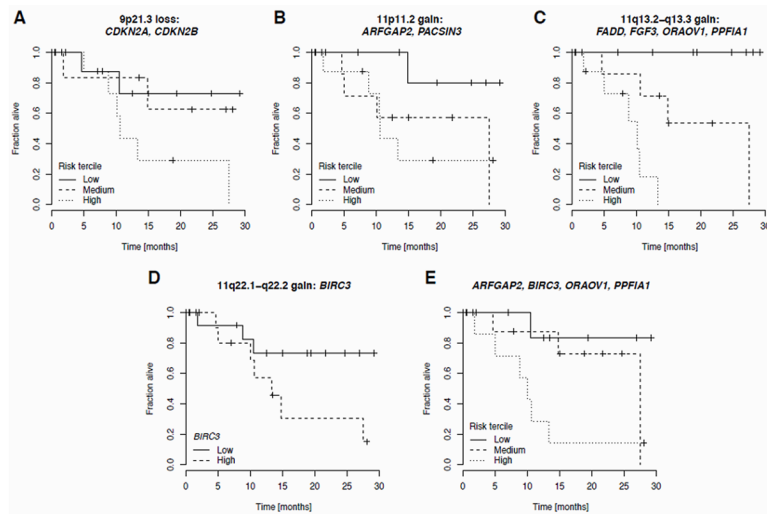


Figure 3.

Kaplan-Meier curves for the survival of GBC cancer patients. A–D: BIC-optimal multivariate Cox proportional hazard model for genes located in focal gains. Patients were split into three risk groups according to the tertiles of the observed hazard function (A–C); in the univariate case (D) high and low expression groups are defined by *BIRC3* expression above and below the median, respectively. E: Combined optimal model based on all genes analyzed in A–D. Multiple gene symbols are listed alphabetically in a panel, so the order implies nothing about their relative importance.

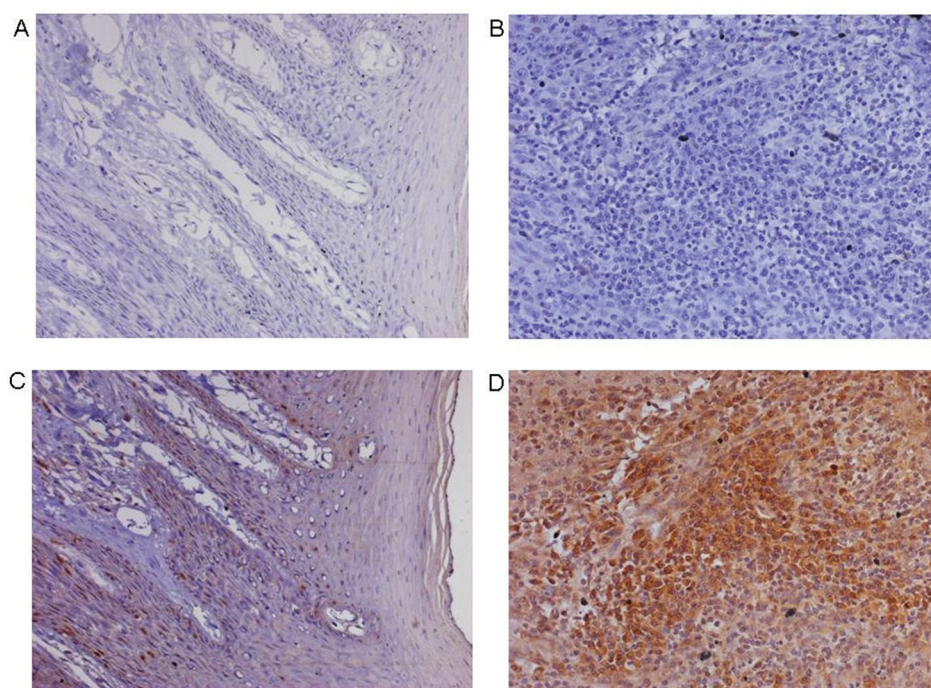


Figure 4. Immunohistochemical analysis of LY6K in normal and tumor tissues of GBC. A – B: represent serum controls for normal and tumor tissues, C: unrelated normal GBC sample showing weak staining; D: GBC cancer showing strong staining with cytoplasmic and membranous localization (200X Magnification).

Table 1

Clinicopathological and demographic details of study cohort

Characteristics	Test Set (n=27) (GE* and qRT-PCR) [†]	Validation Set (n=30) (qRT-PCR) [†]
Gender		
Males	20 (74%)	20 (67%)
Females	7 (26%)	10 (33%)
Age		
Median (IQR) [#]	53 (44–60)	51 (42–56)
Habit profile		
Exclusive Chewers	21 (78%)	22 (73%)
Exclusive Smokers	1 (4%)	2 (7%)
Chewing and Smoking	5 (18%)	6 (20%)
Grade		
Well	0 (0%)	2 (6%)
Moderate	19 (70%)	20 (67%)
Poor	8 (30%)	8 (27%)
Nodal involvement		
Negative (N0)	14 (52%)	15 (50%)
Positive (N+)	13 (48%)	15 (50%)
Stage (pTNM)		
I & II	3 (11%)	0 (0%)
III & IV	24 (89%)	30 (100%)

[†] Shown is the number of cases, except for Age

* GE: Gene Expression analysis;

[#] IQR: Interquartile range