Biomarkers, 2010; 15(1): 39–46

informa
healthcare

ORIGINAL ARTICLE

Expression of angiopoietin-like 4 and tenascin C but not cathepsin C mRNA predicts prognosis of oral tongue squamous cell carcinoma

Zhuomin Wang[1,2], Bo Han[3], Zhuang Zhang[3], Jian Pan[3], and Hui Xia[3]

[1]West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China, [2]The PLA No.452 Hospital, Chengdu, China, and [3]State Key Laboratory of Oral Diseases (Sichuan University), West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China

Abstract

Analysis of gene expression using RNA from the paraffin-embedded tissues is becoming an important way to study cancer pathogenesis. In this article, total RNA was extracted from tissue of 158 cases of paraffin-embedded tongue cancer, and expression of angiopoietin-like 4, tenascin-C and cathepsin-C were detected by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Our results demonstrated that high expression level of angiopoietin-like 4 or tenascin-C was predictive of poor prognosis of tongue cancer patients ($p = 0.024$ and $p = 0.011$, respectively), especially when expression levels of both genes were concomitantly high ($p = 0.001$). Additionally, high expression of angiopoietin-like 4 and tenascin-C, or concomitant high expression of angiopoietin-like 4 and tenascin-C were independent prognostic factors of poor survival in patients with tongue cancer. These results suggest that the improved method of RNA extraction is suitable for analysing gene expression of paraffin-embedded solid tumours. Angiopoietin-like 4 and tenascin-C, especially the combination of angiopoietin-like 4 and tenascin-C, are useful for predicting the prognosis of the patients with tongue cancer, independent of lymph node metastasis status.

Keywords: *Oral tongue squamous cell carcinoma; paraffin-embedded tissue; RNA extraction; RT-PCR; prognosis*

Introduction

The oral squamous cell carcinomas (OSCC) include a diverse group of cancers in which oral tongue squamous cell carcinoma (OTSCC) (Ye et al. 2008) is one of the most common types. Where local invasion and lymphatic dissemination are concerned, OTSCC is more aggressive than other types of OSCC. At present, the most reliable prognostic indicator for OTSCC patients is the status of cervical lymph nodes (Myers et al. 2001, Spiro et al. 1986). The 5-year survival rate was 65% for the pathologically node-negative pN0 group and 29% for the pN+ group (Grandi et al. 1985). The finding of extracapsular spread of cervical lymph node metastasis of OTSCC is associated with even lower rates of survival (Greenberg et al. 2003). However, the status of lymph nodes is an insufficient prognostic parameter, and there are still additional prognostic indicators that affect the clinical outcomes, such as genes involved in lymphatic metastasis of OTSCC.

To find the molecular predictors of OTSCC prognosis, we modelled the metastatic process of tongue cancer cells in nude mice. Using gene microarrays, we obtained the differential gene expression profiles of cancer cells with different potential for metastasis, which provide valuable information that might help in the identification of possible biomarkers that can be used for prognostic prediction of OTSCC. Moreover, in a comprehensive analysis of published datasets on OTSCC gene expression (Suhr et al. 2007, Ye et al. 2008), we found

Address for Correspondence: Zhuang Zhang, State Key Laboratory of Oral Diseases (Sichuan University), West China Hospital of Stomatology, Sichuan University, Chengdu 610041, P.R. China. Tel: +86 28 85501428. Fax: +86 28 85582167. E-mail: zhangzhuang1949@163.com

(Received 13 July 2009; revised 14 August 2009; accepted 14 August 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK Ltd
DOI: 10.3109/13547500903261362

that the expression of angiopoietin-like 4 (*ANGPTL4*), tenascin-C (*TNC*) and cathepsin-C (*CTSC*) was significantly upregulated in OTSCC, and closely associated with OTSCC progression. As a consequence, these genes might also be of value as therapeutic targets and predictors of prognosis in OTSCC.

Comprehensive analysis of gene expression using RNA from fresh or frozen tumour specimens is becoming increasingly important to better understand the cancer progression and prognostication (Chen et al. 2007a, Lossos et al. 2004). Quantitative measurement of gene expression will lead to construction of gene expression-based survival models and identification of possible prognostic factors in the absence of specific antibody (Ludwig & Weinstein 2005). Unlike archived paraffin-embedded tissue samples, frozen tumour specimens are not readily available to carry out clinical studies, and usually have no detailed and complete clinical data. Therefore, analysis of gene expression using RNA from the archived paraffin-embedded tissue is becoming an important way to study cancer prognosis.

However, the degree of RNA degradation and modification during the fixation process varies in different tumour tissue, which may affect the analysis outcome (Cronin et al. 2004, Specht et al. 2001). In this study, we modified a method of RNA extraction and demonstrated its efficiency and reproducibility in OTSCC tissues. Additionally, in order to clarify and validate the prognostic relevance of *ANGPTL4*, *TNC* and *CTSC* in OTSCC, their expression in 158 archived paraffin-embedded specimens of OTSCC was further analysed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in a retrospective study.

Materials and methods

Tissue specimens

Matching formalin-fixed, paraffin-embedded and frozen cancer tissue samples were obtained from eight patients with OTSCC at the West China Hospital of Stomatology. Surgery specimens were fixed in 10% neutral-buffered formalin for 4–6 h before being alcohol dehydrated and embedded in paraffin. Ethical approval was obtained from the Research Ethics Committee of Sichuan University, Chengdu, Sichuan, P.R. China. All specimens were handled and made anonymous according to the ethical and legal standards.

One hundred and fifty-eight archived paraffin-embedded tissue samples were selected randomly from OTSCC patients treated by surgery at the West China Hospital of Stomatology between 1998 and 2001, and the follow-up time was up to 99 months. Clinicopathological data were determined according to the guidelines of the International Union Against Cancer (UICC) (Piemonte 2003): 82 cases were < 60 (51.9%), 76 cases were ≥60 (48.1%); 126 cases were male (79.7%), 32 cases were female (20.3%); 86 cases were N0 (54.4%), 72 cases were N1–3 (45.6%); 93 cases were stage I–II (58.9%), 65 cases were stage III–IV (41.1%); all cases were M0. No previous treatments, including chemotherapy, surgery and radiotherapy were given to these patients.

Total RNA isolation from frozen specimens and paraffin-embedded specimens

To avoid the influence of tumour stroma on the results, the haematoxylin and eosin sections of cancer tissue were observed under the microscope and the margins of OTCC nests were marked. According to the marker, the sections of frozen and paraffin-embedded tissue were carefully trimmed. The percentage of tumour cells in the tumour tissue was about 60–72%.

Frozen tissue samples (50–100 mg) were ground into powder in liquid nitrogen, and then suspended in 1 ml TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). The extraction of total RNA was performed according to the manufacturer's protocol. Briefly, the aqueous phase was used for RNA precipitation with an equal volume of isopropanol. The RNA pellet was washed once with 1 ml 75% ethanol, then air-dried and re-dissolved in appropriate volume of RNase-free water.

Total RNA extraction from paraffin-embedded tissue was performed according to the established method with minor modifications (Masuda et al. 1999). In brief, 10-μm thick sections (about 32 mg tissue) were deparaffinized by ethanol at 37°C. The pellet was allowed to air-dry for 5 min at room temperature, and resuspended in 600 μl of RNA lysis (10 mmol l^{-1} Tris–HCl (pH 8.0), 0.1 mmol l^{-1} EDTA (pH 8.0), 2% SDS (pH 7.3) supplemented with 50 μl of 65 mg ml^{-1} proteinase K) at 60°C for 16–20 h with occasional agitation until the tissue was completely digested. Then, we made a minor change for RNA purification using an equal volume of 70% phenol (pH 4.3):30% chloroform at room temperature twice. After precipitated with an equal volume of isopropanol at -20°C for at least 1 h, the RNA was washed twice in 75% ethanol, dried and re-dissolved in RNase-free water.

Total RNA was quantified spectrophotometrically, and its quality was assessed by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Determination of the length of specific transcripts by PCR

Samples of 2 μg total RNA from paraffin-embedded or frozen tissue were reverse transcribed using the M-MLV reverse transcriptase (Promega BioSciences, San Luis Obispo, CA, USA) according to the manufacturer's

protocol with minor modifications. RNA template and random primers were incubated at 70°C for 10 min to melt the secondary structure within the template and cooled on ice for more than 2 min. Then, the complete reaction mixture was incubated at 30°C for 10 min, 42°C for 60 min and 70°C for 15 min.

To compare the maximal length of RNA transcripts extracted from paraffin-embedded and frozen tissue, we amplified 13 fragments of β-actin ranging in size from 99 to 705 bp. The primers of β-actin were used as previously described, and most primer pairs were designed to span different exons. PCR was performed in a total volume of 25 μl containing 1 μl of reverse-transcribed cDNA. After an initial incubation at 94°C for 5 min, the reaction mixtures were subjected to 35 cycles of amplification using the following procedures: 94°C for 45 s, 55°C for 45 s and 72°C for 45 s, then a final extension step at 72°C for 7 min. PCR products were analysed by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

Quantitative real-time RT-PCR

Real-time RT-PCR was performed using ABI PRISM 7500 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA). The relative expression level of the four housekeeping genes and three target genes were measured using SYBR Green I dye-based method. The sequences of the primers are presented in Table 1. PCR reactions were prepared in a final volume of 25 μl, with a final concentration of 1× Power SYBR Green PCR Master Mix (Applied Biosystems). Thermal cycling comprised of an initial UNG incubation at 50°C for 2 min, AmpliTaq Gold DNA Polymerase activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Each measurement was performed in triplicate and the threshold cycle (C_T) was determined as previously reported (Gloghini et al. 2004).

The differences in average C_T values between the paired paraffin-embedded and frozen specimens were calculated as follows: mean C_T = average C_T (paraffin tissue RNA) – average C_T (frozen tissue RNA). To compare the RNA expression of target genes among different specimens, normalization based on *GUSB* gene expression was performed, and the averages of the normalized C_T values (ΔC_T) were calculated as previously reported (Gloghini et al. 2004, Livak & Schmittgen 2001). Relative mRNA expression of a target gene within a specimen was calculated as $2^{-\Delta CT}$, where $\Delta CT = C_T$(target gene) – C_T(*GUSB*) (Cronin et al. 2004).

Statistical analysis

Statistical analysis was processed using SPSS 13.0 software. Overall survival time was calculated from the date of surgery until death or the last follow-up contact. Kaplan–Meier survival curves and the log-rank test were used to analyse univariate distributions of survival. Cox's proportional hazards multivariate analysis was used to evaluate which factor was the independent prognostic predictor on survival. All *p*-values were given for two-sided testing, and statistical significances were defined as $p < 0.05$.

Results

RNA extraction of OTSCC tissues

To investigate the feasibility of RNA isolation from paraffin OTSCC tissue, we compared the quality and quantity of total RNA separately isolated from paraffin-embedded and frozen OTSCC tissues. Starting from about 32 mg paraffin-embedded tissue, we performed RNA extraction twice with phenol–chloroform, and obtained an average of 40.8 μg (~31.4–53.8 μg) RNA with OD260/280 ratios ranging from 1.9 to 2.0, indicating good RNA quality. To rule out the possibility of DNA contamination and to improve RNA quality, we also tried to add RNase-free DNases in the resolved RNA and incubated at 37°C for 30 min, and then precipitated RNA using the same method. Compared with the DNase-untreated method, we did find significant improvements in the following experiments except for the lower quantity of RNA (data not shown).

RT-PCR amplification of different β-actin fragments in OTSCC tissues

To assess the utility of the extracted RNA to generate longer amplicons by RT-PCR amplification, 13 β-actin

Table 1. Names of genes and primer sequences for the SYBR-Green-based real-time RT-PCR.

Genes	Forward primers	Reverse primers
GUSB	5′-CTCATTTGGAATTTTGCCGATT-3′	5′-CCGAGTGAAGATCCCCTTTTTA-3′
PGK-1	5′-GGGAAAAGATGCTTCTGGGAA-3′	5′-TTGGAAAGTGAAGCTCGGAAA-3′
GAPDH	5′-AGCCGAGCCACATCGCT-3′	5′-TGGCAACAATATCCACTTTACCAGAGT-3′
18S	5′-CGGCTACCACATCCAAGGAA-3′	5′-GCTGGAATTACCGCGGCT-3′
ANGPTL4	5′-GCAGGATCCAGCAACTCTTC-3′	5′-GGTCCAGGAGGCCAAACTGGCTT-3′
TNC	5′-GCACAGTCAAGGAAGTCATTGT -3′	5′- TCCAATTGTGGTGAAGAT -3′
CTSC	5′-GAGACTCTTACCCTGGGAGAT-3′	5′-CCACAGGATGCTTGGTTTCG-3′

amplicons ranging in size from 99 to 705 bp were amplified from the RNA extracted from the paraffin-embedded and frozen specimens. Compared with frozen OTSCC tissues, RNA extracted from the paraffin-embedded OTSCC tissues yielded β-actin amplicons with a similar quality, although at a lower quantity. These results suggested that RNA isolated from paraffin-embedded OTSCC tissues were suitable for gene expression analysis using real-time RT-PCR.

Housekeeping gene expression analysis of OTSCC tissues by real-time PCR

To compare the consistency of quantitative RT-PCR between matched paraffin-embedded and frozen OTSCC tissue samples and to identify the appropriate endogenous control genes for RNA-input normalization in OTSCC tissues, we studied the expression of four commonly used housekeeping genes (*GUSB*, *PGK-1*, *GAPDH* and *18S*), which were selected from among those well known in the literature as commonly constitutively expressed genes across different conditions. The primers amplified amplicons with relatively small sizes (less than 155 bp). As expected, RNA of the endogenous housekeeping genes extracted from the paraffin-embedded specimens were generally lower in quantity (higher C_T) than those extracted from the matched frozen specimens. However, different housekeeping genes exhibited varied expression in different specimens. Of the four endogenous control genes, expression of *GUSB* was the least variable both in the frozen and paraffin-embedded specimens, indicating that *GUSB* was the most appropriate endogenous control gene for the normalization of RNA quality and quantity in OTSCC tissues (data not shown).

Association of clinicopathological factors with overall survival of OTSCC patients

Univariate analysis was carried out to identify those factors significantly associated with overall survival. The results show that p-TNM staging and lymph node metastasis status were significant prognostic factors for overall survival as expected (log rank 24.105 and 24.702, $p < 0.001$ and $p < 0.001$, respectively). Other factors such as age, sex, tumour differentiation were not statistically correlated with overall survival.

Association of ANGPTL4, TNC and CTSC mRNA expression with overall survival of OTSCC patients

ANGPTL4 gene expression levels ranged from 0.16 to 14.71 (median 4.660, mean 4.8213, SD 2.7033, $n = 158$), *TNC* from 0.00 to 0.49 (median 0.102, mean 0.1187, SD 0.0744, $n = 158$) and *CTSC* from 0.010 to 1.160 (median 0.040, mean 0.0736, SD 0.1313, $n = 158$). Cut-off values of each clinicopathological factor were selected according to the median value of continuous variables. We considered that a gene in the cases was overexpressed only when the C_T value was above the median C_T value. Likewise, we considered that a gene in the cases was downregulated when the C_T value was below the median C_T value. The median *ANGPTL4*, *TNC* and *CTSC* gene expression values were used as cut-off values in the univariate model. It was found that median and mean survival times in patients with higher *ANGPTL4* mRNA expression were significantly shorter than that in patients with lower expression (22.0 and 32.7 vs 36.0 and 45.2 months), and that *ANGPTL4* mRNA expression was negatively correlated with overall survival (log rank 5.115, $p = 0.024$, Figure 1A). Analysis of *TNC* mRNA expression showed similar results – median and mean survival times were 19.0 and 32.1, 36.0 and 45.6 months, respectively, in patients with high and low expression (log rank 6.447, $p = 0.011$, Figure 1B). However, no significant correlation between *CTSC* mRNA expression levels and overall survival was found (log rank 0.667, $p = 0.411$, Figure 1C).

Stratifying on lymph node metastasis status, a high expression level of *ANGPTL4* represented a stronger predictive factor in patients with negative lymph node metastasis (log rank 4.642, $p = 0.031$), but not in patients with positive lymph node metastasis (log rank 2.389, $p = 0.122$; Figure 2A, B). However, *TNC* expression showed no statistical significance both in negative and in positive lymph node metastasis patients (log rank 3.120 and 3.141, $p = 0.077$ and $p = 0.076$, respectively), although a trend was observed (Figure 2C, D).

Moreover, it was found that in patients with a high expression level of both *ANGPTL4* and *TNC*, median and mean survival times decreased significantly (18.0 and 25.9 months vs 36.0 and 44.4 months, log rank 10.835, $p = 0.001$; Figure 1D), compared with those patients with a low expression level of one or both genes.

ANGPTL4 and TNC are independent prognostic factors in OTSCC

Multivariate Cox's proportional-hazards regression analysis with stepwise selection was used to evaluate independent prognostic factors associated with survival, and those variables found to be significant at the univariate analysis were used as covariates. The results showed that high *ANGPTL4* and *TNC* expression levels ($p = 0.010$ and 0.021), together with lymph node metastasis status ($p = 0.000$) were independent prognostic factors for survival. Results also showed that concomitant high mRNA expression level of both *ANGPTL4* and *TNC* was a significantly independent prognostic factor ($p = 0.001$).

Discussion

Gene expression detected by DNA arrays or real-time RT-PCR have contributed to finding predictors of survival in human cancers, especially in the absence of specific antibody (Davis & Staudt 2002, van de Vijver et al. 2002). However, the requirement for fresh or snap-frozen tissues has limited their clinical application. Recently, progress has been made with extracting RNA from the formalin-fixed, paraffin-embedded lymphoid tissue and breast cancer tissue (Chen et al. 2007b, Paik et al. 2004), indicating that it may be a more applicable method (Bast & Hortobagyi 2004). However, these techniques are subject to tissue-specific fixation-associated RNA degradation and modification. Thus, optimization of RNA extraction for each kind of tissue is required.

To date, the most successful method for total RNA extraction from paraffin-embedded tissues utilized proteinase K for digestion before acid–phenol–chloroform extraction and carrier precipitation (Chen et al. 2007b, Masuda et al. 1999). This method resulted in significantly better reproducibility and concordance between paired frozen and paraffin-embedded samples especially in lymphoid tissue. However, it was not confirmed that the method of RNA extraction applied in the paraffin-embedded lymphoid tissues could be used in solid tumours, such as OTSCC. In this study, we modified this method by using a higher proteinase K concentration and longer digestion time optimized to 16–20 h. Our data demonstrated that high-yield and high-quality RNA could be isolated, and could be amplified to yield long cDNA fragments (>600 bp). These results indicate that the paraffin-embedded OTSCC tissues may replace frozen tissues for gene expression analysis using real-time RT-PCR, when our modified RNA extraction method is utilized.

During normal or malignant growth, there are no housekeeping genes with constant expression in all tissues (Bustin 2000, Schmittgen & Zakrajsek 2000). Therefore, proper endogenous control genes need to

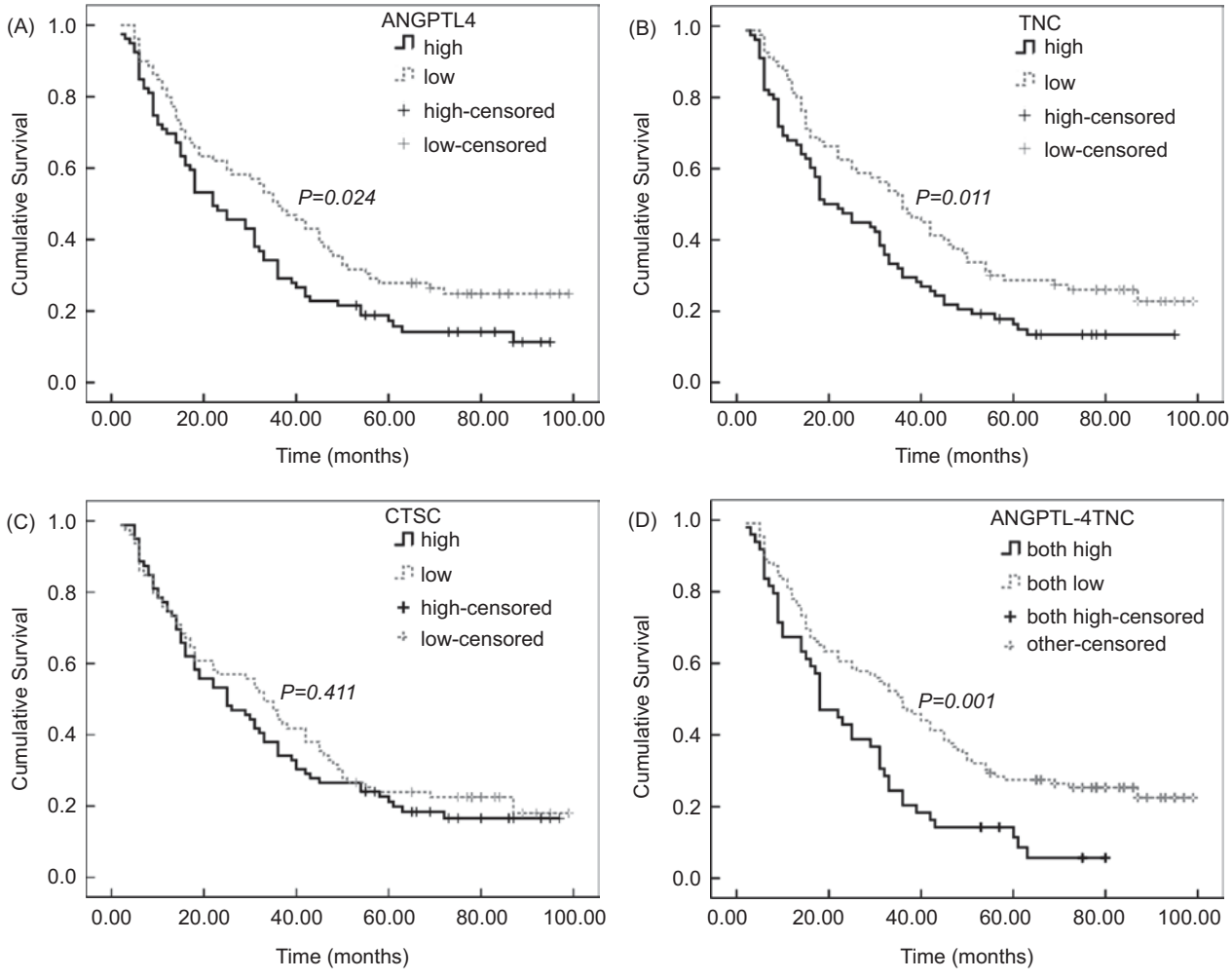

Figure 1. Kaplan–Meier survival analysis for (A) *ANGPTL4*, (B) *TNC*, (C) *CTSC*, and for (D) the combination of *ANGPTL4* and *TNC* in patients with concomitant high expression.

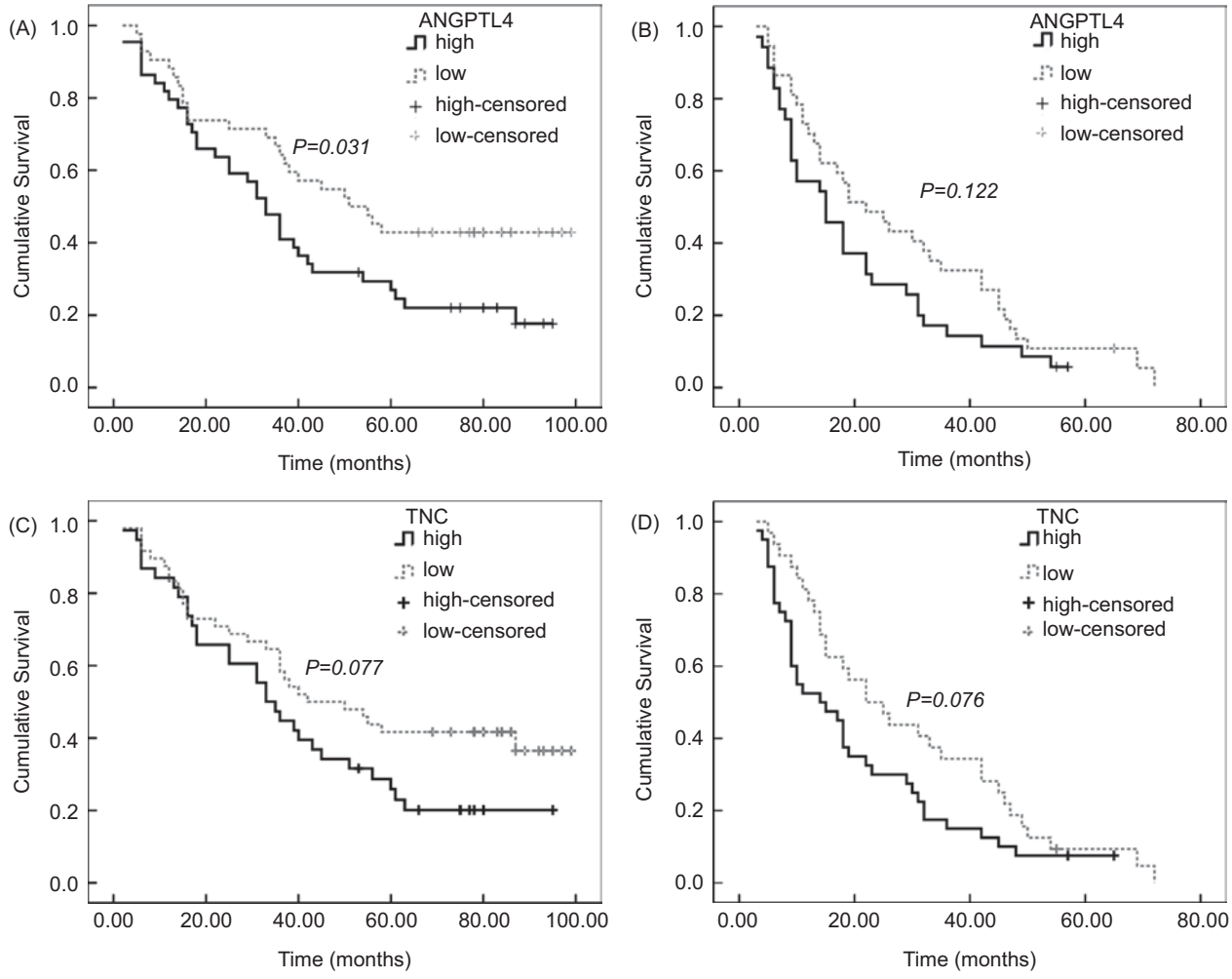

Figure 2. Kaplan–Meier survival analysis of different gene expression levels for negative (A, C for *ANGPTL4* and *TNC*, respectively) and positive (B, D for *ANGPTL4* and *TNC*, respectively) lymph node metastasis patients.

be selected for each cell type and tumour type in each experimental design (Lossos et al. 2003). Here, we chose four housekeeping genes of different abundance, which were widely cited in the literature and exhibited relatively lower variation in expression in different tissues (Chen et al. 2007b, Lossos et al. 2003). Our data suggest that the *GUSB* gene exhibits the lowest variation of expression in paraffin-embedded and frozen OTSCC specimens, and should be used as the suitable endogenous gene for RNA quantity control.

Until now, the relevance of *ANGPTL4*, *TNC* and *CTSC* to development of OTSCC has not been studied in detail. However, previous studies demonstrated that these genes have important roles in the initiation and progression of cancer cells. For instance, *TNC* encodes a large glycoprotein of the extracellular matrix that modulates the biological behaviour of cancer cells in contact with the tenascin-containing environment. During the initiation of oral cancer, TNC could be directly produced by cancer cells and is responsible for the acquisition of an invasive phenotype (Hindermann et al. 1999); ANGPTL4, a regulatory factor of angiogenesis both in tumours and in ischemic tissues, could be produced in hypoxic environments (Ito et al. 2003, Le Jan et al. 2003). Tumour cell-derived ANGPTL4 could disrupt vascular endothelial cell–cell junctions, and facilitates the transendothelial passage of tumour cells, resulting in their subsequent retention in secondary sites (Padua et al. 2008); CTSC plays an active role in protein degradation and proenzyme activation. Its significant upregulation in cancer tissues contributes to metastasis and progression of cancer cell (Dang et al. 2006).Therefore, it is hypothesized that *ANGPTL4*, *TNC* and *CTSC* have a close association with OTSCC progression, especially *ANGPTL4* and *TNC*. *ANGPTL4* and *TNC* might be critical effectors of the OTSCC dissemination, because they could activate the proliferation and migration of cancer cells, and promote

transendothelial passage of tumour cells (Negrini & Calin 2008, Padua et al. 2008).

After the method of RNA isolation was established and the control gene was chosen, we selected *ANGPTL4*, *TNC* and *CTSC* to investigate their relevance with OTSCC prognosis using a real-time RT-PCR method. Before statistical analysis, the data were further processed according to median *ANGPTL4*, *TNC* and *CTSC* gene expression values. It is well known that for every possible cut-off point that you select to discriminate between two groups, some cases with high expression will be correctly classified as 'high', but some cases with high expression will be classified as 'low'. On the other hand, some cases with low expression will be correctly classified as 'low', but some cases with low expression will be classified as 'high'. Different cut-off points used in data analysis might result in the discrepancy. Therefore, we selected the median gene expression value as the cut-off point. In other similar studies, the median gene expression value was also used as the cut-off point, and it was confirmed that this method was effective in ensuring the accuracy of data analysis (Chen et al. 2007a, Lossos et al. 2004).

In our study, univariate survival analysis using the Kaplan–Meier method showed that high expression of *ANGPTL4* was predictive of poor prognosis in OTSCC patients ($p = 0.031$), and patients with high expression levels of *ANGPTL4* had much shorter mean and median survival times compared with those with a low expression level. In comparison, *TNC* showed a similar result ($p = 0.011$), but *CTSC* did not seem to be a prognostic factor for OTSCC patients. For OTSCC patients with negative lymph node, *ANGPTL4* was also a predictor of prognosis. Multivariate analysis using Cox regression also indicated that high expression levels of *ANGPTL4* and *TNC* were independent predictive factors of poor prognosis of OTSCC patients ($p = 0.010$ and $p = 0.021$, respectively). A combination of several molecular markers may increase the predictive power. Univariate and multivariate survival analysis combining *ANGPTL4* and *TNC* showed that concomitant high expression of both genes was a more powerful predictor of poor prognosis in OTSCC patients, with *p*-value of 0.001 and 0.001, respectively.

In short, with the development of RNA extraction and quantification techniques, a large amount of paraffin-embedded tissue specimens provide a great deal of precious material for analysing gene expression, and the information obtained will further enable the realization of individual diagnosis, prognosis prediction and target therapy of OTSCC. In addition, *ANGPTL4* and *TNC* seem to be indicators of OTSCC prognosis, and have a potential role as tools of prognosis estimation and therapeutic targets.

Acknowledgements

We would like to thank Professor Yuming Wen for critical comments on the manuscript. This work was supported by China National Science Foundation grant to Pan Jian, Li Longjiang (contract grant number 30400505, 30471093, and 39700162).

***Declaration of interest*:** The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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