RESEARCH ARTICLE

Prognostic Significance of Desmoglein 2 and Desmoglein 3 in Esophageal Squamous Cell Carcinoma

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Abstract

Objective: Desmogleins (DSGs) are major members among the desmosomal cadherins critically involved in cell-cell adhesion and the maintenance of normal tissue architecture in epithelia. Reports exploring links of DSG family member expression with cancers are few and vary. The aim of this study was to investigate the ratio of DSG2 and DSG3 mRNA expression in esophageal squamous cell carcinoma (ESCC) tissue to normal tissue (T/N ratio) and evaluate correlations with clinical parameters. Methods: The mRNA expression of DSGs, as well as γ-catenin and desmoplakin, was detected by real-time quantitative RT-PCR in 85 cases of ESCC tissue specimens. Results: The expression level of DSG3 mRNA was significantly higher than that of DSG2 in ESCC specimens (p = 0.000). DSG3 mRNA expression highly correlated with histological grade (p = 0.009), whereas that of DSG2 did not significantly relate to any clinicopathologic parameter. Kaplan-Meier survival analysis showed that only DSG3 expression had an impact on the survival curve, with negative DSG3 expression indicating worse survival (p = 0.038). Multivariate Cox regression analysis demonstrated DSG3 to be an independent prognostic factor for survival. Furthermore, correlation analysis demonstrated the mRNA level of DSG3 to highly correlate with those of γ-catenin and desmoplakin in ESCC samples (p=0.000), implying that the expression of desmosomal components might be regulated by the same upstream regulatory molecules. Conclusions: Our findings suggest that DSG3 may be involved in the progression of ESCC and serve as a prognostic marker, while expression of DSG2 cannot be used as a predictor of ESCC patient outcome.

Keywords: Desmoglein - esophageal squamous cell carcinoma - prognosis

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Introduction

Esophageal cancer is a common cancer with an increasing incidence worldwide. Squamous cell carcinoma (ESCC), the most common form of this disease in China, has a well-defined progression from pre-invasive dysplasia to invasive squamous cell carcinoma (Mariette et al., 2003). The process of neoplasm development involves multiple sequential steps including detachment of malignant cells from the primary tumor mass, penetration into blood and/or lymph vessels, transportation in the vessels, attachment to endothelium of distant organs, penetration into secondary host tissue, and formation of new tumor colonies (Chambers et al., 2002). Various cell adhesion molecules including cadherins have been shown to play important roles through this process (Jeanes et al., 2008).

Desmogleins (DSGs) are a family of cadherins consisting of four known subfamily members DSG1, DSG2, DSG3, and DSG4, which show a differentiation-specific expression in epithelia (Kottke et al., 2006). DSG2 is widely expressed in simple epithelia and can be detected in the lower layers of the epidermis, along with high levels of DSG3. DSG1 is prominent in the upper layers, whereas DSG4 is highly represented in the hair follicle. They, together with the desmocollins, constitute the adhesive proteins of the desmosome type of cell-cell junction and take the responsibility for mediating cell adhesion and desmosome formation (Ishii et al., 2001; Garrod et al., 2008).

Reports referring to the link of DSG family member expression with cancer are limited and vary. Some reports have suggest that loss of desmosomal component DSG3 are common events in oral squamous cell carcinoma and head and neck carcinoma and that these events may precede overt malignancy (Wang et al., 2007; Teh et al., 2011). However, another study demonstrates that DSG3 is overexpressed in head neck carcinoma and is a potential molecular target for inhibition of oncogenesis (Chen et al., 2007). In squamous cell carcinomas of the skin, only DSG2 is found to be up-regulated in half of all neoplasms examined and shows a significant higher

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proportion of positive cells in high-risk SCC than in low-risk SCC (Kurzen et al., 2003; Brennan et al., 2009); while in diffuse-type gastric carcinoma, decreased expression of DSG2 is associated with loss of tumor differentiation and poor prognosis (Yashiro et al., 2006). Recently, we have shown that desmocollin-2 (DSC2), the most widely distributed desmocollins family member, plays a causal role in esophageal cellular invasion and metastasis (Fang et al., 2013). DSC2 expression gradually decreases from regions of esophageal hyperplasia to dysplasia to carcinoma in situ, and may serve as a prognostic marker (Fang et al., 2010). However, no data are available in the literature concerning the expression of desmogleins in ESCC. Moreover, the link of each individual member of desmogleins expression to the clinical progression of ESCC is still unknown. Additional studies are needed to understand the expression features of DSGs in ESCC as well as to establish its clinical significance.

In the present study, using a real-time quantitative RT-PCR technique, we studied the correlation of DSG2 and DSG3 mRNA expression in ESCC cases with clinical parameters to evaluate if DSGs expression features are of any prognostic value. These data might provide important information to guide tumor treatments.

Materials and Methods

Patients and samples

ESCC tissue specimens and paired adjacent normal epithelial tissues were obtained from 85 patients (median age, 55 years, range 40-88) who underwent surgery in the Department of Pathology of Shantou Central Hospital from 2007 to 2008. The specimens were immediately frozen in liquid nitrogen following surgery and stored at -70˚C until RNA isolation. All of the tumors were confirmed as ESCC by the Clinical Pathology Department of the Hospital, and the cases were classified according to the 7th edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer (UICC) and were included in this study only if a follow-up was obtained. Patients' data were summarized in Table 1. The study was approved by the ethical committee of the Central Hospital of Shantou City and the Medical College of Shantou University, and written informed consent was obtained from all surgical patients to use resected samples for research.

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was extracted from frozen stored tissues with TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. Reverse transcription was performed in a total volume of 20 μl using 1 μg of total RNA by using the Reverse Transcription System (Promega, USA). The real-time quantitative PCR was carried out on the Rotor-Gene 6000 system (Corbett Life Science, Sydney, Australia). SYBR® Premix Ex TaqTM (TaKaRa) was used according to the manufacturer's instructions. To avoid amplification of contaminating genomic DNA, one of the two primers of each gene were intron spanning. The primers sequence is as follow: For DSG2, the forward primer was 5'-AGCTGCTGTTGCACTGAACGA -3' and the reverse primer 5'- AAGGCAATCTTTGGCTGTGTGA -3'. For DSG3, the forward primer was 5'-CCCAGTTCCTGATGGC TCAGA -3' and the reverse primer was 5'- AAATCGGCT CCATTGGCTGTTA -3'. As an internal control, a fragment of human β-actin was amplified by the following primers: forward 5'- CAACTGGGACGACAT GGAGAAA -3' and reverse 5'- GATAGCAACGTACATGGCTGGG -3'. The PCR conditions were an initial denaturation step of 10 s at 95°C, followed by 40 cycles consisting of 5 s at 95°C, 20 s at 60°C and a 15 sec at 72°C. The relative expression amount was calculated from a relative standard curve obtained by using log dilutions of plasmids containing the gene of interest. Plasmids were constructed by cloning the amplification products into the pGEM-T Vector using the TA-Cloning kit (Promega, USA). The calculated amount of the target genes was normalized to the endogenous reference control gene β-actin. All data are presented as the ratio of the target gene/ β-actin. The status of differentially expressed DSG2 and DSG3 gene in the ESCC tissue was calculated as the ratio of DSG2 and DSG3 mRNA expression in tumor tissue to the adjacent normal tissue (T/N ratio), and defined as 'positive expression' if T/N was > 0.5-fold or 'negative expression' if T/N was≤ 0.5-fold.

Statistical Analysis

Associations of desmogleins with clinicopathological characteristics including age, gender, tumor size, differentiation grade, invasive depth, lymph nodes metastasis, TNM classification were assessed with the Kendall's tau-b test. The extent of DSG2 and DSG3 mRNA expression in ESCC was analyzed by using

Table 1. Survival Information of the Patient by Clinical Characteristics

Parameters	No.	Five Year Survival Rate (%)	*P* Value
Age (year)			
<55	32	53.5	0.081
≥55	53	51.7	
Gender			
Female	21	21.4	0.994
Male	64	58.0	
Tumor size			
≤3cm	25	56.7	0.171
3-5cm	45	50.0	
>5cm	15	36.4	
Differentiation grade			
G1	22	71.8	0.118
G2	54	49.9	
G3	9	22.2	
Invasive depth			
T1+T2	22	64.7	0.228
T3+T4	63	45.1	
Regional lymph nodes			
N0	52	61.7	0.090
N1	33	27.7	
pTNM stage			
IA+IB+IIA+IIB	56	65.5	0.003*
IIIA+IIIB+IIIC+IV	29	21.7	

Note: Statistical analysis: Kaplan-Meier curves (log-rank test) *P Values<0.05 were considered significant

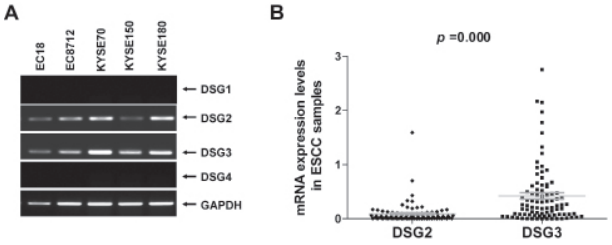

Figure 1. DSG2 and DSG3 Expression Levels in ESCC Cell Lines and Tissue Samples. (A) Representative RT-PCR results of DSG family member (DSG1-DSG4) expression in 5 esophageal cancer cell lines. (B) DSG2 and DSG3 mRNA expression in 85 cases of ESCC tissue samples. The relative expression amount was calculated from a relative standard curve obtained by using log dilutions of plasmids containing the gene of interest. The calculated amount of the target genes was normalized to the endogenous reference control gene β-actin

Table 2. Relationship Between Clinicopathological Features and DSG2 mRNA Expression

Parameters	DSG2 status		*P* Value
	T/N ≤0.5	T/N >0.5	
Age (year)			
≤55	11 (34%)	21 (66%)	0.098
>55	9 (17%)	44 (83%)	
Gender			
Female	5 (25%)	15 (75%)	0.972
Male	16 (25%)	49 (75%)	
Tumor size			
≤3cm	5 (20%)	20 (80%)	0.601
3-5cm	11 (24%)	34 (76%)	
>5cm	4 (27%)	11 (73%)	
Differentiation grade			
G1	5 (23%)	17 (77%)	0.685
G2	12 (22%)	42 (78%)	
G3	3 (33%)	6 (67%)	
Invasive depth			
T1+T2	7 (32%)	15 (68%)	0.216
T3+T4	13 (21%)	50 (79%)	
Regional lymph nodes			
N0	9 (17%)	43 (83%)	0.110
N1	11 (33%)	22 (67%)	
pTNM stage			
IA+IB+IIA+IIB	12 (21%)	44 (79%)	0.536
IIIA+IIIB+IIIC+IV	8 (28%)	21 (72%)	

**P* Values<0.05 were considered significant

a paired sample t-test. Kaplan-Meier curves were constructed for overall survival analysis by a log-rank test. All statistical analyses were performed with SPSS 13.0 software (version 13.0; SPSS, Inc., an IBM Company, Chicago, IL). Each p-value is two-tailed and significance level is 0.05.

Results

Differential expression of DSGs in ESCC cell lines and tissue samples

As desmogleins show a differentiation-specific expression in epithelia (Kottke et al., 2006), we first evaluated each individual member of desmogleins expression in ESCC cell lines. As shown in Figure 1A, RT-PCR showed that mRNA of DSG2 and DSG3 were

Table 3. Relationship Between Clinicopathological Features and DSG3 mRNA Expression

Parameters	DSG3 status		*P* Value
	T/N ≤0.5	T/N >0.5	
Age (year)			
≤55	14 (43%)	18 (57%)	0.210
>55	16 (30%)	37 (70%)	
Gender			
Female	8 (38%)	13 (62%)	0.760
Male	22 (34%)	42 (66%)	
Tumor size			
≤3cm	8 (32%)	17 (68%)	0.916
3-5cm	18 (40%)	27 (60%)	
>5cm	4 (27%)	11 (73%)	
Differentiation grade			
G1	4 (18%)	18 (82%)	0.007*
G2	20 (37%)	34 (63%)	
G3	6 (67%)	3 (33%)	
Invasive depth			
T1+T2	6 (27%)	16 (73%)	0.489
T3+T4	24 (38%)	39 (62%)	
Regional lymph nodes			
N0	18 (35%)	34 (65%)	0.870
N1	12 (36%)	21 (64%)	
pTNM stage			
IA+IB+IIA+IIB	18 (32%)	38 (68%)	0.405
IIIA+IIIB+IIIC+IV	12 (41%)	17 (59%)	

**P* Values<0.05 were considered significant

expressed in all cell lines tested, with DSG3 presented the higher expression levels, while compared with that of DSG2. But for DSG1 and DSG4, there was no signal detectable. To confirm this result, we next examined DSG2 and DSG3 mRNA expression in 85 cases of ESCC tissue samples. The expression level was shown as a ratio between target gene and the reference gene β-actin to correct for the variations in the amounts of RNA. The relative expression amount was calculated from a standard curve obtained by using log dilutions of plasmids containing the gene of interest. Results demonstrated that the expression level of DSG3 mRNA was significantly higher than that of DSG2 in ESCC specimens (p = 0.000, Figure 1B). The mean expression levels of DSG2 and DSG3, calculated for 84 samples studied, were 0.0940±0.1975 and 0.4339±0.5554, respectively.

Correlation between DSG2 and DSG3 mRNA expression and clinicopathologic parameters

To obtain a better understanding of DSG2 and DSG3 mRNA expression in ESCC, the relationships between DSGs and the clinicopathologic characteristics of patients with ESCC were analyzed. With a median follow-up of 40.8 months for the 85 patients analyzed in this study, the mean survival was 42.6 months (range 38.1-47.1), and that the 5-year survival rate was 49.3%. The status of differentially expressed DSG2 and DSG3 gene in ESCC tissues was calculated as the ratio of DSG2 and DSG3 mRNA expression in tumor tissue to the adjacent normal tissue (T/N ratio), and defined as 'positive expression' if T/N was > 0.5-fold or 'negative expression' if T/N was≤ 0.5-fold. By this criterion, 20 (24%) patients had a negative

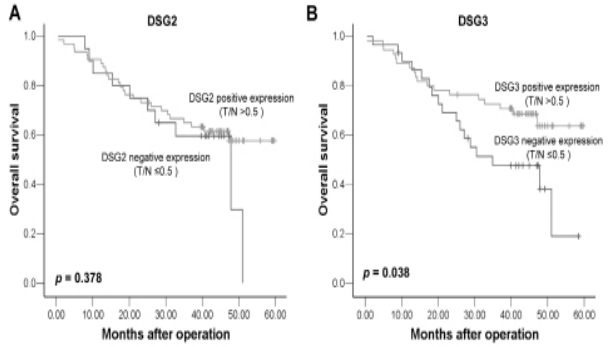

Figure 2. Kaplan-Meier Estimates of the Overall Survival by DSG2 and DSG3 Status in 85 Pairs of ESCC Tumor and Corresponding Non-tumor Samples. The Kaplan-Meier analyses of DSG2 (A) and DSG3 (B) mRNA expression in 85 patients with ESCC illustrate that only DSG3 expression levels had an impact on the survival curve, with negative DSG3 mRNA expression (T/N ≤0.5) indicating worse survival (p=0.038)

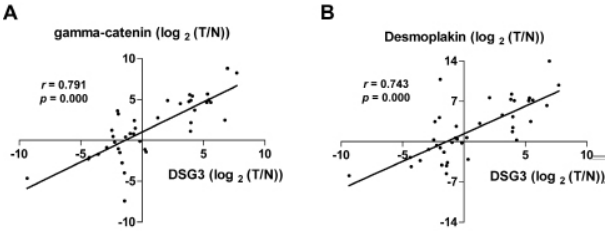

Figure 3. DSG3 mRNA Expression Levels Correlate with that of γ-catenin (A) and Desmoplakin (B) in ESCC. Pearson's correlation analysis was performed to investigate the correlation of these desmosomal components in 40 pairs of ESCC tumor and corresponding non-tumor samples

DSG2 expression status and 65 (76%) patients had a positive DSG2 expression status; while 30 (35%) patients had a negative DSG3 expression status and 55 (65%) patients had a positive DSG3 expression status. Table 2-3 shows associations between the clinicopathologic characteristics of patients with ESCC and DSG2 or DSG3 expression status. A significant correlation was observed between DSG3 expression status and histologic grades of tumors (p=0.007), whereas the expression of DSG2 did not significantly correlate with any clinicopathologic parameters. Positive DSG3 expression cases (T/N > 0.5) were found in 82% of grade I, 63% of grade II, and 33% of grade III. ESCC patients with histologic grade III were more likely to have negative DSG3 expression (T/N ≤ 0.5). There were no significant correlations between DSG3 expression levels and other clinical parameters, such as pTNM classifications and stages grouping, in patients with ESCC.

Prognostic implications of DSG2 and DSG3 mRNA expression

The expression level of DSG2 and DSG3 were next evaluated for association with survival time using Kaplan-Meier method. The results showed that only DSG3 expression had an impact on the survival curve, with negative DSG3 expression indicating worse survival (p=0.038). Among 85 cases of ESCC patients, in 30 cases of negative DSG3 expression (T/N ≤ 0.5), the median survival time was 51.4 months and the 5-year survival rate was 19.1%, whereas in 55 cases of positive DSG3 expression (T/N > 0.5), the median survival time was 68.6 months and the 5-year survival rate was 63.7% (Figure 2). The use of the Cox regression model in multivariate analysis showed that DSG3 expression status was an independent prognosis predictor (p=0.021) (Table 4).

Table 4. Univariate and Multivariate Cox Regression Analysis by DSG3 Expression Status

Univariate	Mean time to survival (months)	Percentage of survival (95% confidence interval)	*P* value
T/N ≤0.5	33.974±3.499	19.1 (29.117-42.831)	0.042*
T/N >0.5	45.795±2.813	63.7 (40.281-51.309)	
Multivariate	**Relative Risk**	**95% confidence interval**	***P* value**
Regional lymph nodes	1.801	0.916-3.543	0.088
DSG3	0.446	0.225-0.884	0.021*

**P* Values<0.05 were considered significant

DSG3 mRNA expression levels correlate with that of γ-catenin and desmoplakin in ESCC

The general picture that has emerged over the past decade is that the desmosomal cadherins mediate desmosome type of cell-cell adhesion and bind directly to γ-catenin. γ-catenin is thought to interact with desmoplakin and thereby link the cadherin tails to the intermediate filament network (Garrod et al., 2008). To evaluate the concordance of these desmosomal components in ESCC samples, we next analyzed the relationship between DSG3 and γ-catenin or desmoplakin mRNA expression (Figure 3). Positive correlations between DSG3 and γ-catenin or desmoplakin mRNA expression were observed in the 40 cases of ESCC samples (r = 0.791, p = 0.000; and r = 0.743, p = 0.000, respectively). These results imply that the expression of desmosomal components might be regulated by the same upstream regulatory molecules.

Discussion

This study is one of the first attempts to evaluate the expression of desmogleins in ESCC and the link of each individual member of desmogleins expression to the clinical progression of ESCC. Down-regulation of desmosomal proteins has been suggested to be a sign of reduced adhesiveness in metastasizing cells (Green et al., 2007). However, all prior studies in cancer report contradictory results (Kurzen et al., 2003; Yashiro et al., 2006; Chen et al., 2007; Wang et al., 2007; Brennan et al., 2009; Teh et al., 2011). We demonstrate in here that DSG3 may be involved in the progression of ESCC and serve as a prognostic marker; while the expression of DSG2 cannot be used as a predictor for ESCC patient outcomes.

We have examined each individual member of desmogleins mRNA expression in ESCC cell lines; this shows differences in the expression of subsets of desmogleins genes. It remains unknown whether such different molecular signatures of desmogleins confer different adhesion strength and cellular activity. The lack of DSG1 and DSG4 expression in cancer cell lines, are shown consistently in the current study and also by others (Tada et al., 2000; Wu et al., 2003). These two

desmogleins are expressed in the upper compartment of the epidermis and hair follicle, respectively. This indicates their association with epidermal barrier function and hair development (Kottke et al., 2006). Thus, it is plausible that different pathways are involved in the regulation of desmogleins expression in ESCC.

By quantitative assays that measured mRNA, we also observe a clear difference in DSG2 and DSG3 expression in ESCC tissue specimens. The expression level of DSG3 mRNA is significantly higher than that of DSG2 (p = 0.000). Analysis of literature on molecular components of desmogleins and their association with tumorigenesis reveals that coordination of expression of these molecules in tumor cells is often altered (Dusek et al., 2011). Our further study shows that DSG3 mRNA expression highly correlated with histological grade and patient survival, whereas the expression of DSG2 does not significantly correlate with any clinicopathologic parameters. Recent study suggests that specific desmosomal cadherins contribute differently to overall adhesive strength and tissue integrity. In basal cell carcinoma, DSG2-mediated adhesion appears to be more proliferation-associated, whereas DSG3-mediated adhesion seemingly is more differentiation-associated (Gornowicz-Porowska et al., 2011), while in keratinocytes, DSG2, when compared to DSG3, is less important for cell-cell adhesion but is required for keratinocyte cohesion under conditions of increased mechanical stress (Hartlieb et al., 2013). Our findings imply that specific desmogleins contribute differently to the development of ESCC and that DSG2 compared to DSG3 is less important in this context.

It has been suggested that desmosomal components may contribute to the differentiation of human endometrial carcinoma (Nei et al., 1996). Besides, Kyrodimou et al. (2013) and Gómez-Morales et al. (2013) report that DSG3 is a marker for poorly differentiated OSCC and also for squamous differentiation in non-small-cell carcinomas. Consistent with these results, we observe that lose of DSG3 expression is associated with poor differentiation in ESCC, but not with lymphatic metastases and depth of tumor invasion, suggesting that the reduction of DSG3 expression is associated with loss of the ability of adhesion, as previously reported (Hartlieb et al., 2013). Desmosomal proteins have also been considered as prognostic markers in various cancer types. For example, downregulation of desmoplakin expression provides prognostic information in human oropharyngeal cancer (Papagerakis et al., 2009). Decreased DSG3 expression is associated with poor prognosis in lung cancer (Fukuoka et al., 2007). Recently, we report that desmocollin 2 expression level is an independent prognostic factor for ESCC (Fang et al., 2010). In this study, we find that negative expression of DSG3 is associated with an unfavorable prognosis in ESCC patients. Like DSC2, DSG3 seem to be a potential prognostic marker in ESCC.

There is still a very interesting issue of what mechanisms allow desmosomal adhesion and desmosomal components (particularly DSGs) dysfunction to promote tumor progression. The disturbance of desmosomal adhesion can result in tissue integrity damage and possibly induction of tumor cell migration and proliferation (Dusek et al., 2011). Our present correction analysis, in which both DSG3 and γ-catenin or desmoplakin mRNA expression display positive correlations, implies that the expression of desmosomal components might be regulated by the same upstream regulatory molecules. Although we does not determine which pathways or factors are involved in the regulation of desmosomal components expression in ESCC. Our recent study shows that miR-25 up-regulation in esophageal carcinomas promotes cell migration by inhibiting the expression of DSC2 (Fang et al., 2013). Is miR-25 to be responsible for other desmosomal components regulation in ESCC? which need to be identified. Moreover, further research may show whether the expression levels of certain desmosomal protein coding gene or perhaps an expression level network of several desmosomal genes might serve as a new biomarker in ESCC.

In summary, we observe a clear difference in DSG2 and DSG3 expression in ESCC tissue specimens. The expression level of DSG3 mRNA is significantly higher than that of DSG2. DSG3 may be involved in the progression of ESCC and serves as a prognostic marker. To our knowledge, this is the first study to investigate the expression of these two desmosomal molecules and their associations to survival in ESCC. Further studies are needed to explore the functional role of DSGs in ESCC carcinogenesis.

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