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Main Article

Overexpression of *Cap43* gene in supraglottic laryngeal squamous cell carcinoma

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Abstract

Objective: This study aimed to determine the expression of the *Cap43* gene in supraglottic laryngeal squamous cell carcinoma, and to evaluate any correlation between *Cap43* gene expression and tumour-associated macrophage infiltration.

Methods: Four human head and neck squamous cell carcinoma cell lines were cultured (Hep2, KB, Ca9-22 and HSC-3) and expression of the *Cap43* gene was analysed by Western blotting. In addition, paraffin-embedded samples of supraglottic laryngeal squamous cell carcinoma and normal supraglottic laryngeal mucosa from 84 patients were analysed immunohistochemically using antibodies to *Cap43* and cluster of differentiation 68 glycoprotein. Patients' clinical status was compared with their immunohistochemical results.

Results: All four head and neck squamous cell carcinoma cell lines exhibited *Cap43* expression. The Hep2, Ca9-22 and HSC-3 cells showed a markedly higher level of *Cap43* protein than the KB cells. A statistically significant difference was found in *Cap43* expression, comparing different differentiation levels and comparing different metastasis stages, for supraglottic squamous cell carcinoma. The number of tumour-associated macrophages correlated with expression of *Cap43*, not only in the tumour area ($r = 0.3708$, $p = 0.0005$) but also in the peritumour area ($r = 0.2847$, $p = 0.0087$).

Conclusion: In supraglottic laryngeal squamous cell carcinoma, overexpression of the *Cap43* gene is associated with tumour differentiation and acts an important suppressive factor in the process of tumour metastasis. The *Cap43* gene may be a cancer-specific marker. High expression of the *Cap43* gene appeared to correlate with infiltration of tumour-associated macrophages.

Key words: Supraglottic Laryngeal Cancer; *Cap43*; Tumour Associated Macrophages; CD68

Introduction

Cancer is a polygenetic disease. A variety of genetic alterations are known to affect the aggressiveness, stage and histological grade of laryngeal squamous cell carcinoma (SCC).

The differentiation-related gene 1 (Drg-1) gene, which codes a 43-kDa protein, is a nickel- and calcium-inducible gene which is also known as *Cap43* (43-kDa protein induced by free intracellular calcium), NDRG1 (N-myc downstream-regulated gene 1) and Rit43 (reduced in tumour).[1–4] The gene is mapped to human chromosome 8q24.2. The *Cap43* gene possesses three unique, 10 amino acid tandem repeats at the C-terminal end.[4,5] It has often been observed to be up-regulated during cell differentiation or cell growth arrest, and may be an important malignancy-related factor. The *Cap43* gene is generally accepted to be an interesting candidate for molecular biology studies of carcinogenesis. Expression of the *Cap43* gene within infiltrating macrophages has been discussed in association with the differentiated or activated status of monocytes and macrophages.[6] Increased expression of the *Cap43* gene within a cancerous region appears to depend on macrophage infiltration. Studies of the *Cap43* gene in renal, prostate, colon and breast cancers have shown that it may be involved in the growth, differentiation and metastasis of tumour cells.[7,8] The *Cap43* gene is also more highly expressed in cancerous regions than in non-cancerous regions.[9] However, there is currently little information on the expression of the *Cap43* gene in human laryngeal SCC.

In laryngeal carcinoma, the invading pattern of the primary tumour is particularly important. Supraglottic tumours tend to be more advanced and histologically more aggressive than glottic tumours, and their prognosis is also poorer. It has been reported that

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supraglottic tumours have a worse prognosis than their glottic counterparts due to the anatomy of the larynx.[10,11] For example, the supraglottic site has profuse lymphatics, and hence offers an easier pathway for tumour invasion, while the glottic site has elastic layers and sparse lymphatics, which might hinder tumour spread. However, previous molecular studies of supraglottic and glottic laryngeal SCC have neglected the influence of such anatomical factors.

The present study first analysed the expression of *Cap43* protein in four human head and neck SCC cell lines. The study then went on to assess the correlation between *Cap43* expression and patients' clinical staging and tumour-associated macrophage infiltration, in patients with supraglottic laryngeal SCC.

Materials and methods

Patients and samples

The study enrolled an initial cohort of 108 patients with supraglottic laryngeal cancer who were treated at Kurume University Hospital between October 1995 and October 2004. Twenty-four patients who did not undergo surgery or who received radiotherapy before surgery were excluded. Therefore, the actual analysis was done on 84 patients who underwent total laryngectomy either with or without radical or modified radical neck dissection. Seventy-seven patients were men and seven were women. Patients' ages ranged from 43 to 95 years (mean, 67 years). Histopathologically, all patients had SCC (well differentiated in 15 patients, moderately differentiated in 48 and poorly differentiated in 21). According to the 2002 tumour–node–metastasis (TNM) classification, patients were classified as follows: T_1, four patients; T_2, 22; T_3, 17; T_4, 41; N_0, 50; N_+, 34 (N1, six; N_2, 27 and N_3, one); M_0, 71; and M_1, 13. During the follow-up period, recurrence was detected in 11 patients; follow up was uneventful in the remaining 73 patients. The overall patient survival rates were 70.72 per cent at three years and 65.74 per cent at five years.

Histopathological findings for the 84 patients were initially screened at a low magnification, and cancerous as well as non-cancerous tissue was extracted from the original paraffin blocks for further study. In order to provide a control for this study, normal supraglottic laryngeal mucosa tissue was obtained from five patients who had undergone total laryngectomy for advanced tongue cancer.

Cell culture

The following four human head and neck SCC cell lines were cultured: laryngeal SCC (Hep2; TKG0403; Tohoku, Sendai, Japan); oral SCC (KB; TKG0401; Tohoku); gingival SCC (Ca9-22; TKG0485; Tohoku) and tongue SCC (HSC-3; TKG048; Tohoku). Each of the four cell lines was cultured in Dulbecco's modified Eagle's medium supplemented with 10 per cent fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C in a 5 per cent CO_2 atmosphere, in 100-mm dishes. Cells were grown at a subconfluent density (75–90 per cent) and were evaluated by Western blot analysis.

Antibodies

A polyclonal rabbit antibody against *Cap43* protein was kindly provided by the Department of Medical Biochemistry, Kyushu University, and used at a dilution of 1:5000 for Western blot analysis and 1:500 for immunohistochemical staining. Monoclonal mouse anti-human cluster of differentiation 68 glycoprotein (1:100, M0876; Dako, Tokyo, Japan) was used to label the macrophages.

Western blot analysis

The cells were rinsed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer (890 μl mammalian protein extraction reagent, 100 μl 10 per cent phosphatase inhibitor, 10 μl 1 per cent phenylmethylsulfonyl fluoride (PMSF), 1 μl aprotinin and 1 μl leupeptin). The cell lysates, containing equal amounts of protein (30 μg), were separated by polyacrylamide gel electrophoresis at 120 V for 2 hours until the bromophenol blue tracking dye in the samples reached the bottom of the gel, and were then transferred to Immobilon membrane. This membrane was incubated with blocking solution (5 per cent skim milk) for 1 hour, probed with anti-*Cap43* antibody for 2 hours and with a second anti-rabbit antibody for 1 hour. The membrane was then placed in a chemiluminescent working solution (500 μl supersignal west Femto luminol peroxide buffer + 500 μl supersignal west Femtol stable peroide buffer) for 2 minutes.

Immunohistochemical analysis

Three-micrometre thick, consecutive sections were placed on slides, deparaffinated and dehydrated in xylol with graded alcohol. After rinsing with phosphate-buffered saline, the sections were placed in hot 10 mmol/l citrate buffer (pH 6.0) and heated in a microwave oven (twice, 5 minutes each) for antigen retrieval. The EnVision immunohistochemistry method was utilised to stain for *Cap43* and cluster of differentiation 68 glycoprotein. Using a Dako TechMate TM Horizon automated immunostainer, the slides were incubated with primary antibodies for 60 minutes. Dako EnVision TM system-peroxidase was used during the EnVision immunohistochemistry staining process, and diaminobenzidine substrate chromogen solution was applied, followed by counterstaining with haematoxylin.

Determination of expression

For Western blot analysis, the intensity of luminescence was quantified using a charge-coupled device (CCD) camera combined with an image analysing system (Irlas-1000 Life, Fuji Film, Tokyo, Japan). Coomassie brilliant blue staining of protein was used to confirm equal amounts of sample loading. The breast cancer cell lines SKBr-3 and T47D were used as a positive and negative control, respectively.

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Using a digital optical microscope (VHX-200; Keyence, Osaka, Japan) at ×400 magnification, expression was evaluated by an independent investigator blinded to the patient's history. The degree of *Cap43* expression was categorised into two groups according to the percentage of positive cells, either less than 20 per cent (i.e. weak expression) or 20 per cent or more (strong expression). Cluster of differentiation 68 glycoprotein positive cells (i.e. tumour-associated macrophages) were counted separately in the tumour and peritumour areas. The degree of infiltration of tumour-associated macrophages was described as either numerous (i.e. ≥ 10) or minority groups (i.e. <10). In addition, co-expression of *Cap43* and cluster of differentiation 68 glycoprotein was examined using a consecutive series of sections.

For immunohistochemical analysis, sections of tongue SCC served as positive controls. Sections incubated without the primary antibody served as negative controls.

Statistical analysis

Any correlation between immunohistochemical results and clinicopathological features was assessed using the chi-square test and Student's *t*-test. Any correlation among the groups of parameters was evaluated using the Spearman test and linear regression analysis. Actuarial survival curves were plotted using the Kaplan–Meier method. Patients were dichotomised to weak and strong groups according to the intensity of *Cap43* expression. Outcomes in different groups were compared using the log-rank and chi-square tests. A p value of less than 0.05 (two-sided) was considered to indicate statistical significance.

Results

Cap43 protein levels were examined in four head and neck SCC cell lines by Western blot analysis. Figure 1 shows that all four head and neck SCC cell lines exhibited an immune reaction to *Cap43*. The Hep2, Ca9-22 and HSC-3 cells showed a markedly high level of *Cap43* protein, whereas the KB cells showed a weak reaction.

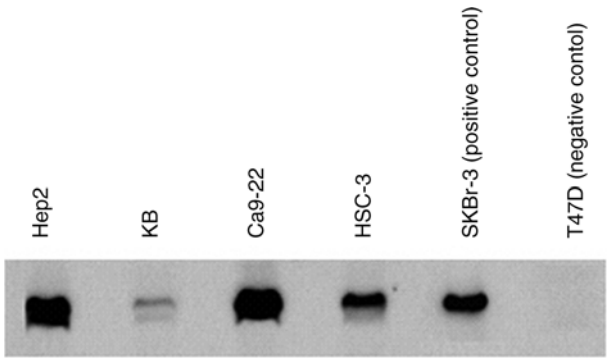

FIG. 1

Western blot analysis for *Cap43* protein in four human head and neck squamous cell carcinoma lines (Hep2, KB, Ca9-22 and HSC-3; SKBr-3 was used as a positive control and T47D as a negative control). See text for details of analysis.

The *Cap43* expression was analysed in patients' cancer tissue, and was detected in 59 of 84 patients (70.24 per cent). The mean percentage of positive cells in each individual ranged from 0 to 100 per cent (36.40 per cent). Normal laryngeal mucosa from the same patient did not show any *Cap43* staining positivity, nor did laryngeal mucosa from the five tongue cancer patients. Positive staining for *Cap43* was found in the cell nuclei (18 cases), cytoplasm (22 cases, Figure 2b) or membrane (10 cases, Figure 2c). Seven cases showed a positive staining pattern in both the nuclei and cytoplasm, and three cases showed a positive reaction in all parts of the cell (Figure 2d). However, the *Cap43* expression pattern showed no correlation with differentiation, N stage, metastasis, survival status or recurrence. There was no significant difference among the areas of the cell nuclei, cytoplasm, and membrane.

The relationship between *Cap43* expression and the patients' various clinical features is summarised in Table I. A statistically significant difference in *Cap43* expression was found, comparing different differentiation levels and comparing different metastasis stages (Spearman's $r = 0.2739$, $p = 0.0012$). The average percentage of *Cap43*-positive cells was higher in the M_0 group ($38.25 \pm 4.27\ n$) compared with the M_1 group ($26.30 \pm 11.60\ n$), and the difference was remarkably statistically significant ($t = 6.67$, $p = 0.0000$). However, no other correlation was found for *Cap43* expression, regarding T stage, N stage, prognosis or recurrence status.

Using the same tissue specimens, the distribution of cluster of differentiation 68 glycoprotein positive cells (i.e. tumour-associated macrophages) was also evaluated in the tumour and peritumour areas. In general, the number of cluster of differentiation 68 glycoprotein positive cells was greater in the peritumour areas compared with the tumour areas (61.23 ± 8.88, 39.81 ± 6.75, respectively; $t = 2.60$, $p = 0.0111$; Table II). In the peritumour areas, cluster of differentiation 68 glycoprotein positive cells were significantly more predominant in well or moderately differentiated carcinomas ($p = 0.046$). The number of cluster of differentiation 68 glycoprotein positive cells was greater in the peritumour areas compared with the tumour areas (Figure 3). The number of cluster of differentiation 68 glycoprotein positive cells correlated with tumour differentiation in both the tumour and peritumour areas. In addition, the level of cluster of differentiation 68 glycoprotein positive cells in the tumour areas demonstrated a significantly negative correlation with survival status ($\chi^2 = 4.50$, $p = 0.034$), while the same result was not found in the peritumour areas. The number of cluster of differentiation 68 glycoprotein positive cells in the tumour regions of the surviving and deceased patient groups was also compared; the number in the surviving group was lower than that in the deceased group. The number of cluster of differentiation 68 glycoprotein positive cells infiltrating the tumour nests was significantly

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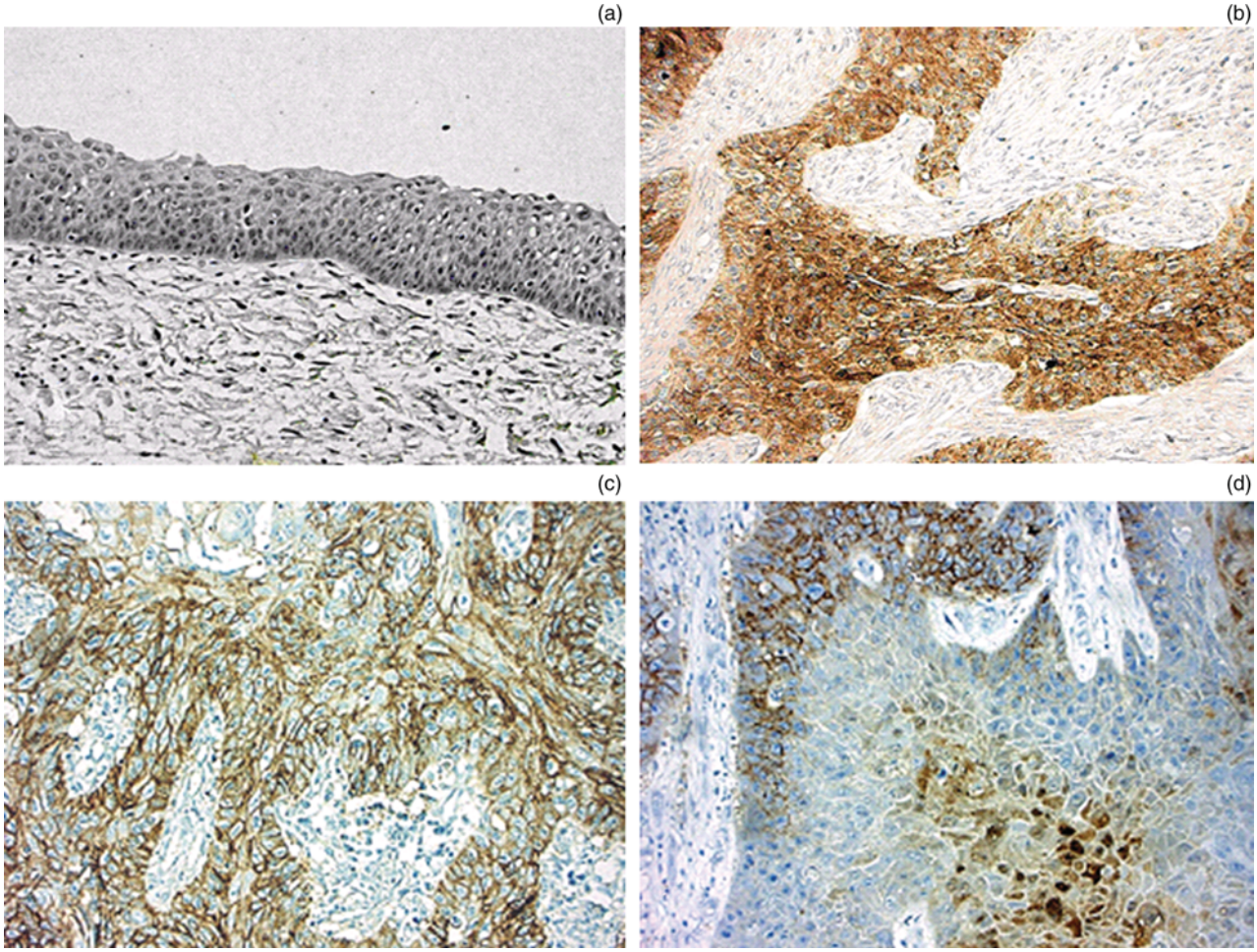

FIG. 2

Photomicrographs showing immunohistochemical staining for *Cap43* protein in normal supraglottic mucosa and supraglottic laryngeal squamous cell carcinoma (SCC). (a) Normal supraglottic tissue, showing negative staining for *Cap43*. (b) Cancerous tissue from supraglottic SCC, showing positive staining for *Cap43* in a cytoplasmic pattern. (c) Cancerous tissue from supraglottic SCC, showing positive staining for *Cap43* in a membranous pattern. (d) Cancerous tissue from supraglottic SCC, showing positive staining for *Cap43* in a cytoplasmic, membranous and nuclear pattern (×400).

statistically associated with T stage, while in the peritumour regions it was significantly statistically associated with N stage. Infiltration of cluster of differentiation 68 glycoprotein positive cells in supraglottic SCC is summarised in Table II.

The number of cluster of differentiation 68 glycoprotein positive cells was significantly associated with *Cap43* expression, in both the tumour and peritumour areas ($r = 0.3708$, $p = 0.0005$; $r = 0.2847$, $p = 0.0087$; Figures 4a and b). Samples with numerous cluster of differentiation 68 glycoprotein positive cells showed strong staining of *Cap43* (Figures 5a and 4b).

Discussion

The *Cap43* gene was cloned from human lung carcinoma A549 cells in 1998. It was thought to be a novel gene induced by nickel compounds.[12] The *Cap43* gene is expressed in all of the tested human cell lines and in three human cell lines under hypoxic conditions.[3,13] Intracellular Ca^{2+} is also essential for *Cap43* gene expression.[13] The *Cap43* gene codes for a 3.0-kb messenger ribonucleic acid segment encoding a Mr 43000 protein. This protein has not been found in the cell nucleus, but has been found localised in the perinuclear region and cytoplasm.[1] However, almost all *Cap43* gene studies have indicated that the cell nucleus also expresses the protein, as observed in the current study.[14] At present, the molecular mechanism of such differential expression is unknown.

Expression of the *Cap43* gene has been extensively reported in the fields of prostate, breast and renal cancer.[8,9,15] In colon, breast and prostate cancer, *Cap43* gene expression is relatively low in cancerous tissue compared with non-cancerous tissue. At present, there have been no previous reports on *Cap43* expression in head and neck tumours. Contrary to the findings of previous studies of human laryngeal carcinoma, the present study found *Cap 43* positive cells neither in the normal, control specimens nor in the noncancerous tissue of cancer patients. Similar results have previously been reported by Nishie *et al.* for renal cell carcinoma; the *Cap43* gene was found to be highly expressed in

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TABLE I

RELATIONSHIP BETWEEN *CAP43* EXPRESSION AND CLINICAL PARAMETERS

Parameter	Pts (*n*)	*Cap43* expression		*p*
		Weak	Strong	
Tumour differentiation				0.013*
Well	15	5	10	
Mod	48	20	28	
Poor	21	16	5	
T stage				0.871
T_{1-2}	30	15	15	
T_{3-4}	54	26	28	
N stage				0.478
N_0	50	26	24	
N_{1-3}	34	15	19	
M stage				0.027*
M_0	71	31	40	
M_1	13	10	3	
Recurrence?				0.376
No	73	37	36	
Yes	11	4	7	
Survival				0.988
Alive	61	31	30	
Dead	23	10	13	

*$p < 0.05$. Pts = patients; mod = moderate; T = tumour; N = node; M = metastasis

cancerous regions compared with noncancerous regions.[9] Cangul *et al.* evaluated *Cap43* gene expression in cancerous and normal tissue from 13 kinds of human tissue, and observed various degrees of *Cap43* gene expression in cancer cells, depending on tissue type.[14–16] The reason for the overexpression of *Cap43* protein in laryngeal cancer cells is not clear. Hypoxia is thought to limit tumour growth, and tumours with poor vascularisation fail to grow and metastasise.[17] On the other hand, the presence of hypoxia results in more aggressive cancer phenotypes which are associated with a poorer prognosis.[18] During the early carcinogenesis of supraglottic laryngeal cancer, hypoxia develops because of inadequate vascularisation, thereby inducing the expression of high levels of the *Cap43* protein.

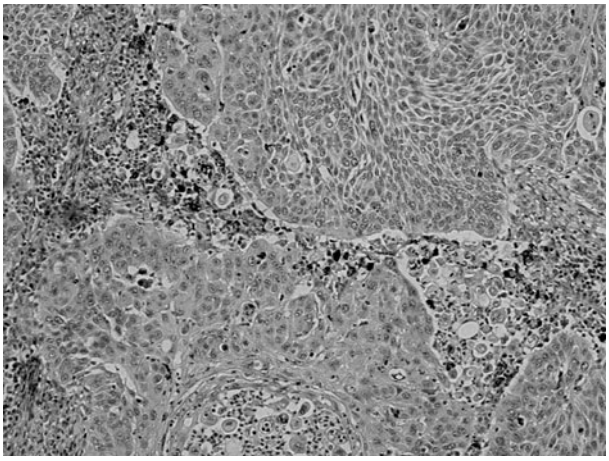

FIG. 3

Photomicrograph showing immunohistochemical staining for cluster of differentiation 68 glycoprotein positive (CD68 +) cells in supraglottic squamous cell carcinoma, showing CD68+ cells predominantly infiltrating the peritumour areas, and less so in the tumour areas (×400).

TABLE II

INFILTRATION OF CD68+ TUMOUR-ASSOCIATED MACROPHAGES INTO TUMOUR AND PERITUMOUR SUPRAGLOTTIC SCC TISSUE, BY CLINICAL PARAMETER

Parameter	Pts (*n*)	Tumour		Peritumour	
		CD68+ TAMs (PTEN)	*p*	CD68+ TAMs (PTEN)	*p*
Tumour differentiation			0.0000*		0.0000*
Well	15	61.93 ± 76.92		57.87 ± 82.57	
Mod	48	42.77 ± 65.78		81.91 ± 90.55	
Poor	21	17.24 ± 24.99		16.33 ± 18.53	
T stage			0.0120*		0.3397
T_{1-2}	30	17.30 ± 29.11		49.77 ± 84.91	
T_{3-4}	54	52.31 ± 71.23		67.59 ± 79.56	
N stage			0.1129		0.0089*
N_0	50	30.98 ± 42.34		42.28 ± 55.13	
N_x	34	52.79 ± 81.58		89.09 ± 104.08	
M stage			0.2091		0.5201
M_0	71	36.17 ± 54.06		58.76 ± 76.12	
M_x	13	59.69 ± 94.19		74.69 ± 108.89	
Recurrence?			0.3878		0.2461
No	73	37.53 ± 63.31		57.20 ± 79.32	
Yes	11	54.91 ± 50.51		87.91 ± 94.21	
Survival			0.0069*		0.1430
Alive	61	28.61 ± 50.19		53.21 ± 80.58	
Dead	23	69.48 ± 81.69		82.48 ± 81.69	
Total	84	39.81 ± 6.75		61.23 ± 8.88	0.0111*

*$p < 0.05$. CD68 + =cluster of differentiation 68 glycoprotein positive; SCC = squamous cell carcinoma; pts = patients; TAMs = tumour-associated macrophages; mod = moderate; T = tumour; N = node; M = metastasis; PTEN-phosphate and tensin homologue deleted on chromosome 10

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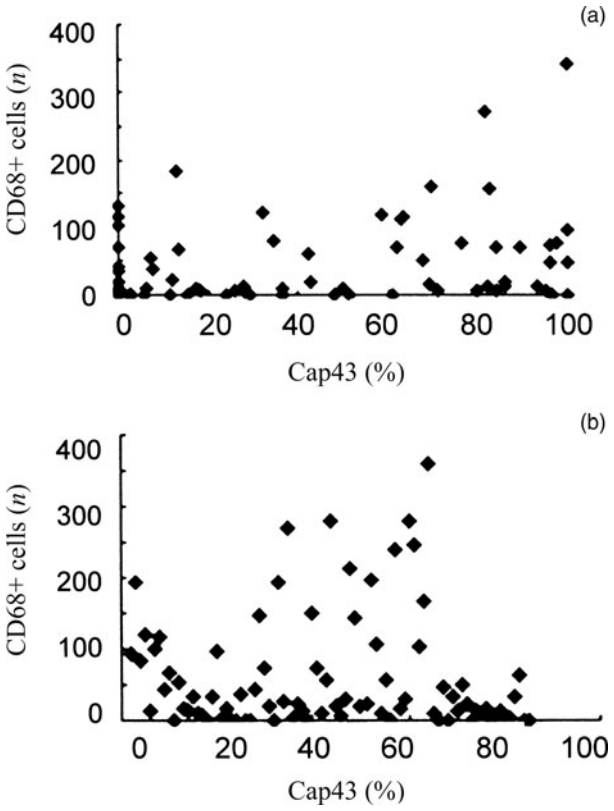

FIG. 4

Correlation between expression of *Cap43* protein and infiltration of cluster of differentiation 68 glycoprotein positive (CD68 +) tumour-associated macrophages. There was a significantly positive correlation between the two factors, both (a) in the tumour nest area ($r = 0.3708$, $p = 0.0005$) and (b) in the peritumour area ($r = 0.2847$, $p = 0.0087$).

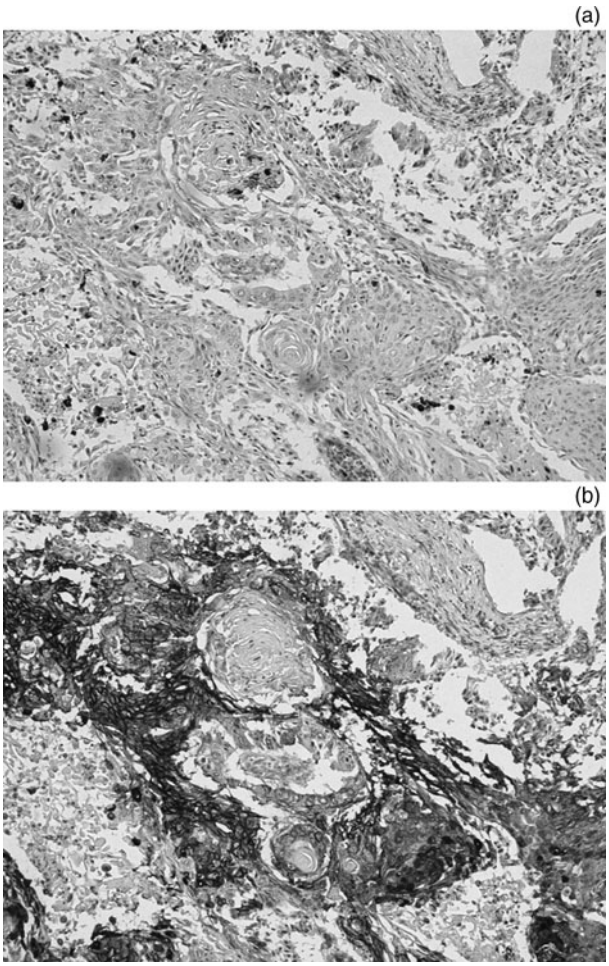

FIG. 5

Photomicrographs showing immunohistochemical staining for cluster of differentiation 68 glycoprotein positive (CD68 +) cells in supraglottic squamous cell carcinoma. (a) Representative tissue showing infiltration of CD68+ tumour-associated macrophages in the cancerous area. (b) Strong *Cap43* staining co-localised with strong CD68+ staining (as seen in 5a) (×400).

The *Cap43* gene is considered to be a differentiation-related gene and to correlate with the differentiation of several tumours. The gene has also been shown to exert a similar metastasis-suppressive effect in breast and prostate cancer.[8,19] In the current study, there was a significant difference in *Cap43* expression level between the metastasis and non-metastasis groups, and this is consistent with the hypothesis that *Cap43* may be involved in inhibition of metastasis. These results raise the possibility that *Cap43* may lie 'downstream' of the mitogen-activated protein kinase kinase-4 pathway, because *Cap43* is up-regulated by the tumour suppressors PTEN and p53, thereby leading to metastasis suppression.[19]

Carcinogenesis and progression is an extremely complicated process. The *Cap43* gene is controlled by multiple factors and is responsive to various cellular and molecular conditions. The biochemical function and exact downstream target of *Cap43* in tumour promotion and progression is not yet fully understood. In renal cell carcinoma, Nishie and colleagues have found high *Cap43* gene expression in areas of macrophage infiltration.[9] In the current study, infiltration of tumour-associated macrophages was evaluated by antibody staining for cluster of differentiation 68 glycoprotein, and numerous cluster of differentiation 68 glycoprotein positive cells were detected not only in the tumour area but also in the peritumour area (where strong staining for *Cap43* was also present). Tumour-associated macrophages are thought to support tumour progression and metastasis by producing angiogenetic factors, growth factors, cytokines, and proteases such as vascular endothelial growth factors A, C and D, interleukin 8 and tumour necrosis factor α. Tumour-associated macrophages may modulate not only haemangiogenesis but also lymphangiogenesis.[20,21] Piquemal *et al.* suggested that differentiation of human monocyte cells in response to phorbol ester results in increased expression of the *Cap43* gene.[6] In the current study of supraglottic SCC, numerous tumour-associated macrophages were found in tissue of peritumor area from patients with a poor prognosis. This association of poorer prognosis with tumour-associated macrophages may partially be due to angiogenesis and lymphangiogenesis.[22] The *Cap43* gene may therefore play a role in the infiltration and differentiation of tumour-associated macrophages.

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Conclusion

The present study explored the significance of the *Cap43* gene in supraglottic laryngeal SCC. Expression of the *Cap43* gene was associated with tumour differentiation and metastasis, and was related to the infiltration of tumour-associated macrophages. Further study is thus required to elucidate how the *Cap43* gene suppresses metastasis, and to investigate the molecular mechanism by which the *Cap43* gene enhances intratumoural macrophage infiltration.

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