Cross-reactivity between *Candida albicans* and Oral Squamous Cell Carcinoma Revealed by Monoclonal Antibody C7

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Abstract. *Background: Monoclonal antibodies developed against Candida albicans cell wall mannoproteins cross-react with human ovarian cancer. These antibodies reacted with the nuclear pore complex protein Nup88, which is overexpressed in a number of human tumors. The aim of this study was to investigate if Nup88 revealed by monoclonal antibody C7 is overexpressed in early oral squamous cell carcinoma (EOSCC) and if this expression has a prognostic value. Patients and Methods: A monoclonal antibody against a C. albicans cell wall manoprotein was used to investigate the expression of Nup88 in 34 EOSCC (T1/T2 N0M0). Results: Mab C7 was mostly located in the cytoplasm and extracts from EOSCC showed specific bands of 47-40 and 70 kDa that were not observed in normal oral mucosa. The highest levels of Mab C7 reactivity were observed in 13 (38.2%) tumors. The Kaplan-Meier test showed the median survival time to be shorter in those EOSCC cases with the highest Mab C7 reactivity. Conclusion: The monoclonal antibody C7 raised against a C. albicans cell wall mannoprotein cross-reacts with an antigen from oral squamous cell carcinoma whose expression is associated with poor prognosis. The overexpression of this antigen is associated with a poor prognosis in early squamous cell carcinoma.*

Oral squamous cell carcinoma (OSCC) is the most prevalent malignancy of the oral cavity, constituting over 90% of cases. In the Basque Country, there is a high incidence of oral cancer compared to other Spanish or European regions (1). The 5-year survival rate for OSCC has remained at approximately 50% for the past several decades. Early diagnosis and treatment are essential to achieve a good prognosis. Some of the molecular alterations associated with oral carcinogenesis could be related to the prognosis (2).

The aetiology of OSCC is multifactorial and a number of carcinogenic agents such as tobacco and alcohol have been described (3). The existence of a link between the colonization or infection by *Candida albicans* and the development of OSCC has been suggested in some studies (4-6). It has been reported that *C. albicans* was a promoter of oral mucosal neoplasia in a model of oral carcinogenesis (5) and the amount of oral yeasts has been correlated with the presence and degree of oral epithelial dysplastic or neoplastic change in oral mucosa (6).

C. albicans induces potent immunological responses in immunocompetent hosts (7). In general, these cellular and humoral responses are directed at controling fungal multiplication. However, some of the antibodies produced may react with host tissues due to cross-reactivity between fungal and host antigenic determinants. Cross-reactivity between *C. albicans* and ovarian antigenic determinants, as well as between Candida and tumoral cells, has been described (8-10). We have previously reported that two monoclonal antibodies (Mabs) C6 and PA10F developed against *C. albicans* cell wall mannoproteins cross-react with human ovarian cancer (10). Subsequently, we found that Mab C6 reacted with the nuclear pore complex protein Nup88, which is overexpressed in a number of human tumors (11-14). Virtually all tumors studied expressed Nup88, whereas their benign counterparts did not (11, 12). Mab C7, closely associated with Mab C6 (10, 15) also reacts with Nup88 and has been selected for its strong reactivity with ovarian cancer tissue (16).

The aim of this study was to investigate if Nup88 revealed by Mab C7 is overexpressed in early oral squamous cell carcinoma (EOSCC) and if this expression has a prognostic value.

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Key Words: Oral squamous cell carcinoma, antigenic expression, Nup88, monoclonal antibody C7, immunohistochemistry, prognosis.

Patients and Methods

Patients and tumor samples. The experimental protocols were approved by the Institutional Review Board of the School of Medicine and Odontology at the University of the Basque Country/EHU, and all the patients gave informed consent prior to participation. MabC7 staining in 34 surgical specimens of EOSCC (Stages I and II) collected from the tongue and floor of the mouth from 34 patients (29 men and 5 women) was examined. The clinical stage was evaluated according to the TNM classification proposed by UICC (17) and included 8 T1N0M0 cases and 26 T2N0M0 cases. The patients' median age was 55.7 years (from 41 to 81 years). In all cases, clinical and histological data were collected according to a previous protocol and the follow-up was 5 years. The histopathological grading of malignancy was obtained according to a multifactorial system (18). The tumor thickness was measured (19) and the percentage of keratinized cells was counted (20). All cases were treated initially with surgery.

Immunohistochemistry. The immunohistochemical procedure has been described in previous publications (10-14). Mab C7 was used diluted at 1:100. As negative controls, slides were similarly processed but omitting the primary antiserum. The sections immunostained were assessed by three independent observers. Ten tumor fields were chosen and hyperplastic epithelium at the margins was excluded. A scoring system which took into account both the staining intensity and the proportion of reactive tumor cells, and gave a score of high expression (showing intense staining in more than 50% of the tumor cells) or low expression (showing negative or weak staining or in less than 50%) was adopted (Figure 1).

Monoclonal antibodies. Mab C7 was produced following standard methods with splenocytes from BALB/c mice immunized by intraperitoneal injections of a *C. albicans* mannoprotein (15). Monoclonal antibody against Nup88 was purchased from BD Biosciences (Palo Alto, CA, USA).

Western-blotting. Two samples from tumor tissue and normal oral mucosa from two cancer-free volunteers were homogenized in a buffer containing 0.4% sodium dodecyl sulphate (SDS) and beta-mercapto-ethanol (5%). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (21) in 10% acrylamide gels. The total amount of protein loaded was 15 μg per lane. After electrophoresis, the proteins were transferred to a nitrocellulose membrane in a semi-dry blotter at 10 W for 10 min. The Mab C7 was used diluted 1:200. A Mab against actin (Sigma Chemical Co., St. Louis, MO, USA) was used to confirm that the protein content of the different lanes was equivalent.

Statistics. The association between antigenic expression and the clinicopathological data was assessed using the Chi-square test. The correlation between survival time and expression of Nup88 in OSCC was assessed according to the Kaplan-Meier test. A $p<0.05$ was considered statistically significant.

Results

Table I shows the main clinicopathological data in relation to Mab C7 staining.

Table I. *Relationship between Mab C7 staining and clinicopathological features.*

	Mab C7 staining		
	High n=13	Low n=21	*p*
Tumor site			
Tongue	8 (61.5%)	13 (61.9%)	
Mouth floor	5 (38.5%)	8 (38.1%)	1.000
Size			
T1	2 (15.4%)	6 (28.5%)	
T2	11 (84.6%)	15 (71.5%)	0.444
Tumor thickness			
<8 mm	3 (23.1%)	12 (57.2%)	
>8 mm	10 (76.9%)	9 (42.8%)	0.052
Grade of malignancy			
>2	6 (46.2%)	8 (38.1%)	
<2	7 (53.8%)	13 (61.9%)	0.643
% Keratinized cells			
>10%	5 (38.5%)	15 (71.5%)	
<10%	8 (61.5%)	6 (28.5%)	0.058
Perineural invasion			
Yes	7 (53.8%)	4 (19.1%)	
No	6 (46.2%)	17 (80.9%)	0.035*
Recurrence			
Yes	9 (69.2%)	8 (38.1%)	
No	4 (30.8%)	13 (61.9 %)	0.078
Survival			
Yes	4 (30.7%)	14 (66.6%)	
No	9 (69.2%)	7 (33.3%)	0.042*

*Statistically significant.

The average grading of malignancy was 1.93 (1.29-2.86) and the mean primary tumor size was 2.72 cm. The average tumor thickness was 7.9 mm (1.8-13.68 mm) and the mean percentage of keratinized cells was 10.19% (1.5-44.75 %).

The Mab C7 reactivity was mostly located in the cytoplasm (Figure 1A). Mab C7 reacted strongly with OSCC tissue, but normal oral mucosa surrounding the tumoral lesions showed absence of Mab C7 reactivity (Figure 1B). Mab C7 was highly stained in 13 (38.2%) out of the 34 cases. A higher staining score was observed in the group with greater tumor thickness (>8 mm) ($p=0.052$) and also in the group that showed a lower percentage of keratinized cells (<10%) ($p=0.058$). The group in which tumors recurred showed higher antigen expression than the group who had no recurrence ($p=0.078$). There were statistically significant differences between high and low expression levels in survival at 5 years' follow-up ($p=0.042$) and the presence of perineural invasion ($p=0.035$).

The Kaplan-Meier test showed median survival time to be shorter in EOSCC patients showing the highest Mab C7 reactivity compared with those showing low levels of Mab C7 reactivity (log-rank $p=0.075$) (Figure 2).

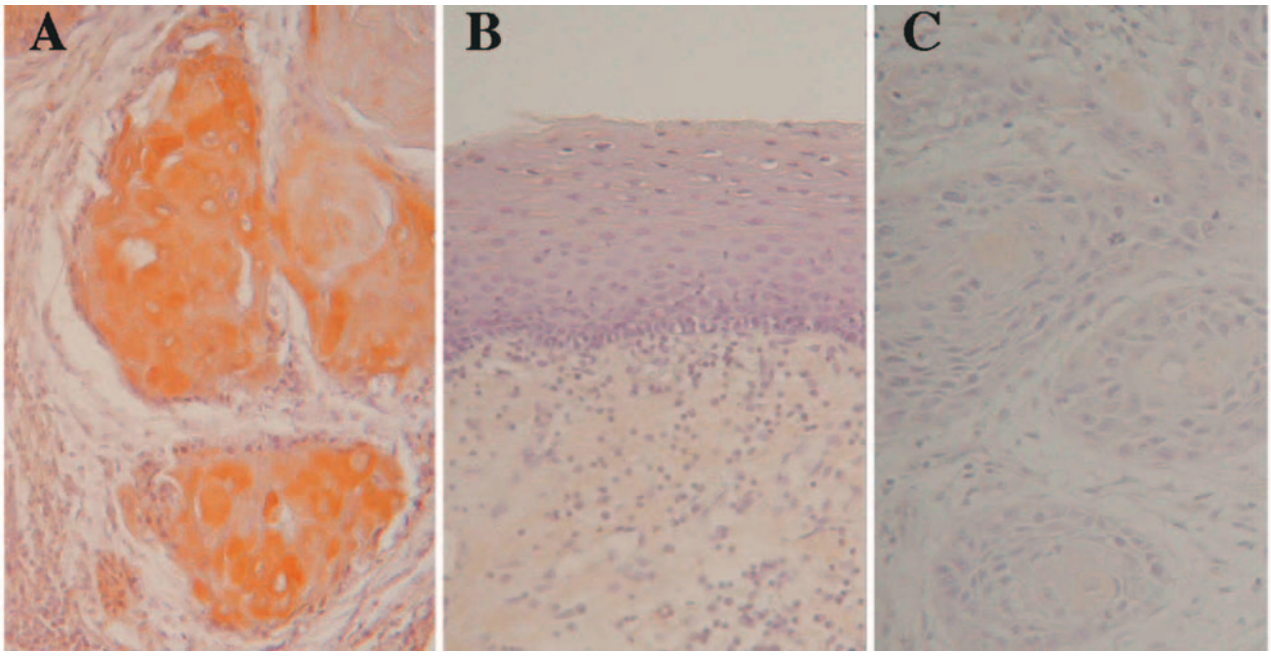

Figure 1. *Immunohistochemical analysis of Nup88 expression in OSCC using Mab C7. A) representative section showing strong C7 immunoreactivity. B) Section of normal oral mucosa from the same patient as in panel A showing absence of Mab C7 staining, and C) section of a lesion showing weak Mab C7 staining. The chromogen was DAB. Original magnification x40.*

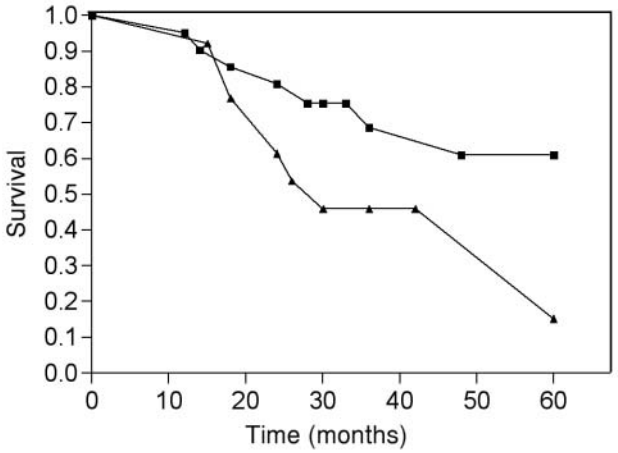

Figure 2. *Kaplan-Meier survival curves of patients with OSCC showing high (▲)and low or no MabC7 staining (■).*

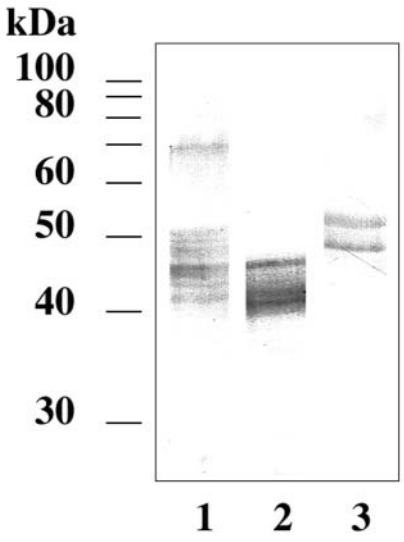

Figure 3. *Western blots of 10% slab gels loaded with OSCC extracts (lanes 1 and 2) and a normal human oral mucosal extract (lane 3) treated with Mab C7. Molecular weights of standard proteins are listed to the left of the gel.*

Immunoblot analysis of selected samples with Mab C7 strongly corroborated the specificity of the immunohistochemistry findings, since extracts from OSCC showed bands of 40-47 kDa that were not observed in normal mucosa (Figure 3). The extract from one of the patients also showed a specific band of 70 kDa. No reactivity was observed by immunoblot with Mab against Nup88 (data not shown).

Discussion

Mab C7 showed a high specificity for OSCC tissue and was evident in the cytoplasm of tumoral cells. Since Nup88 is a nucleoporin, the observed reactivity suggested an overproduction of the protein by the tumoral cell and its shedding into the cytoplasm (16, 22). The specificity may

be attributed to the bands of 70 and 40-47 kDa present in extracts from the OSCC patients that were not observed in normal oral tissue. Since Mab C7 has been shown to react with a recombinant Nup88 protein (16), the lack of reactivity with a component of 88 kDa in the human tissues studied suggested that the bands of 70 and 40-47 kDa represent degradation products from the native protein. The labile nature of Nup88 protein in human tissues was confirmed by the lack of staining shown by the commercially available Mab against Nup88. As reported by the manufacturer, the Mab only reacts with a band of 88 kDa in HeLa extracts (16).

Nup88 is one of the nucleoporins from vertebrate nuclear pore complexes, structures that form channels that connect the cytoplasm and the nucleus, and which control nucleocytoplasmic exchange (23). One of the recently described properties of Nup88 is its relation to cancer. After being related to two variants of leukemia (24, 25), its overexpression has been reported in human ovarian carcinoma (11) and in a number of epithelial tumors from the stomach, colon, liver, pancreas, breast, lung, ovary, uterus, prostate and kidney, as well as in different mesenchymal and miscellaneous tumors, but not in benign tumors or hyperplasias (12). A previous study (26) showed that Nup88-mRNA expression was related to all clinical and biological parameters associated with a worse prognosis in breast cancer, and that this effect was specific for Nup88 and not for nucleoporins in general. Another recent (27) study also related Nup88 expression to the development, aggressiveness and differentiation of colorectal tumors. Data presented in this paper are in agreement with those studies. Although we have not found a statistical correlation between overexpression of Nup88 and all of the clinicopathological variables, a tendency was observed with some variables that are considered indicative of aggression such as tumor thickness, less differentiation, high recurrence and the presence of perineural invasion. The patients with tumors showing Nup88 overexpression had a statistically significant lower survival time during the follow-up period of five years. These results are also in agreement with those of Zhang *et al.* (13) and Emterling *et al.* (14) who, using other antibodies, reported that enhanced expression of Nup88 was linked to increased tumor aggressiveness.

In conclusion, we found that a monoclonal antibody raised against a *Candida albicans* cell wall mannoprotein cross-reacted with an antigen from oral squamous cell carcinoma. This antigen may be Nup88 whose expression is associated with poor prognosis. Further studies are required to confirm the prognostic value of the expression of this protein in other stages of oral cancer and in oral precancer.

Acknowledgements

Financial support: Grants PI020432 and PI021271 from Fondo de Investigación Sanitaria, PIPR–02-19 from Universidad Rey Juan Carlos, UPV05/86 and 9/UPV0093.327-13550/2001 from Universidad del País Vasco/EHU and PM99-0033 from the Spanish Ministry of Education and Culture.

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Received April 17, 2007
Revised June 5, 2007
Accepted June 8, 2007