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ΔNp63 Overexpression, Alone and in Combination with Other Biomarkers, Predicts the Development of Oral Cancer in Patients with Leukoplakia

Pierre Saintigny,[1] Adel K. El-Naggar,[3] Vali Papadimitrakopoulou,[1] Hening Ren,[1,4] You-Hong Fan,[1] Lei Feng,[2] J. Jack Lee,[2] Edward S. Kim,[1] Waun Ki Hong,[1] Scott M. Lippman,[1] and Li Mao[1,4]

Abstract

Purpose: The risk of malignant transformation of oral preneoplastic lesion (OPL) is difficult to assess. ΔNp63 is an early oncoprotein associated with mucosal tumorigenesis. The purpose of this study was to assess ΔNp63 expression in OPL and its role as a marker of oral cancer risk.

Experimental Design: ΔNp63 expression was determined using immunohistochemistry in 152 OPL patients included in a clinical trial comparing retinyl palmitate alone or plus β-carotene with low-dose 13-*cis*-retinoic acid. The associations between ΔNp63 expression as well as ΔNp63 expression with other potential risk factors for oral cancer development were analyzed.

Results: ΔNp63 expression was positive in 41 (27%) patients, clusters of intraepithelial inflammatory cells (EIC) were noted in 37 (26%) patients, and podoplanin (previously reported) was positive in 56 (37%) patients. Significantly more patients whose lesions were ΔNp63 positive or exhibited EIC developed oral cancers. In the multicovariate analysis including age, treatment, and histologic status as cofactors, positive ΔNp63 expression was associated with an increased hazard ratio of 3.308 (95% confidence interval, 1.663-6.580; $P = 0.0007$). Patients whose lesions showed positive ΔNp63, podoplanin, and EIC had the highest oral cancer risk with a hazard ratio of 4.372 (95% confidence interval, 1.912-9.992; $P = 0.0005$) and 61% oral cancer development rate at 5 years compared with 15% of other OPL patients ($P < 0.0001$).

Conclusion: ΔNp63 overepression in OPL is associated with increased oral cancer risk. Together, ΔNp63, podoplanin, and EIC may be used as biomarkers to identify OPL patients with substantially high oral cancer risk. (Clin Cancer Res 2009;15(19):6284–91)

Oral cancer is common worldwide. Leukoplakia is the most commonly diagnosed oral preneoplastic lesion (OPL) in the oral cavity (1), with a rate of malignant transformation between 17% and 24% during periods of up to 30 years (2–4). Prevention of malignant transformation is particularly important in view of the poor prognosis associated with oral squamous cell carcinoma (5). Clinical predictors of malignant transformation in oral leukoplakia have been recently reviewed (6). Although they are important, they are nonspecific and interdependent to some degree. Lesions with dysplastic features remain the most important indicator for risk of oral cancer in the population. However, the predictive value of dysplasia is insufficient, and more important, the majority of oral cancers develop from lesions that lack dysplastic changes (2, 3, 7).

Few prevention studies have shown efficacy in preventing malignant transformation of leukoplakias (8). One of the major difficulties in OPL prevention trials is identification of patients with higher cancer risk (9). Toward this need, we recently showed that podoplanin, together with histologic status, was a promising biomarker for predicting the risk of oral cancer development (10). To further improve our risk assessment model, we are exploring additional molecular markers using the same patient population.

We selected ΔNp63 in this study because it is a homologue of the p53 tumor suppressor (11) and frequently amplified and overexpressed in squamous cell carcinomas, including head and neck squamous cell carcinoma (HNSCC; refs. 12–15). In normal oral mucosa and reactive epithelial hyperplasia, ΔNp63 expression is only observed in the basal layer, with

Authors' Affiliations: Departments of [1]Thoracic/Head and Neck Medical Oncology, [2]Biostatistics and Applied Mathematics, and [3]Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; and [4]Department of Oncology and Diagnostic Sciences, University of Maryland Dental School, Baltimore, Maryland

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Requests for reprints: Li Mao, Department of Oncology and Diagnostic Sciences, University of Maryland Dental School, 650 West Baltimore Street, Baltimore, MD 21201. Phone: 410-706-4339; Fax: 410-706-6115; E-mail: lmao@umaryland.edu.

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Translational Relevance

Oral leukoplakia is the most common oral premalignancy (OPL), but the risk of malignant transformation is difficult to assess. In this study, we show for the first time that overexpression of ΔNp63 in OPL is associated with increased oral cancer risk in a large population from whom OPL samples had been collected in a prospective longitudinal manner. Together, ΔNp63 expression, podoplanin expression, and presence of clusters of intraepithelial inflammation cells are strong predictors of increased oral cancer risk and may be used as biomarkers in assessing oral cancer risk in patients with OPL. This work has never been reported in any previous abstract, presentation, report, or publication.

gradually decreased expression at the middle of the epithelium. But in dysplastic lesions, the expression may extend to the middle spinous layer and in some cases to almost the full thickness of the dysplastic epithelium (16–18). p63 has two major isoforms, Tap63 and ΔNp63 (11), and each has multiple variants due to alternative splicing at the COOH terminus. In general, the Tap63 variants behave like p53, whereas the ΔNp63 variants possess oncogenic properties (11). However, the role of such deregulated expression in cancer risk assessment is unknown.

The primary objective of this study was to determine, in a relatively large population from whom OPL samples had been collected in a prospective longitudinal manner, whether considering overexpression of ΔNp63 alone or in combination with other molecular and morphologic features can be associated with a high risk to develop oral cancer.

Materials and Methods

Patients and specimens. All of the 162 randomized and eligible patients who were enrolled in a randomized chemoprevention trial at The University of Texas M. D. Anderson Cancer Center were eligible for this study. From 1994 to 2001, the patients had been diagnosed with OPL and randomly assigned to intervention with 13-*cis*-retinoic acid (13cRA) versus β-carotene (BC) + retinyl palmitate (RP) versus RP alone. Formalin-fixed, paraffin-embedded biopsy specimens were obtained at enrollment or after enrollment but before any event (defined as the diagnosis of oral cancer). Clinicopathologic parameters were obtained from the clinical trial database. The follow-up data were obtained from a combination of chart review and a telephone interview. More detailed clinical information has been previously described by Papadimitrakopoulou et al. (19). The study was approved by the institutional review board, and written informed consent was obtained from all patients.

Tissue processing and immunohistochemistry. Tissue sections (4 μm thick) from formalin-fixed, paraffin-embedded tissue blocks of OPL were mounted on positively charged glass slides. ΔNp63 immunostaining was done using the avidin-biotin peroxidase complex technique, as described previously (10). Briefly, slides were deparaffinized and rehydrated. To retrieve antigenicity, the slides were steamed with 10 mmol/L citrate buffer (pH 6.0; DakoCytomation) for 30 min. The slides were then incubated in 10% fetal bovine serum for 30 min at room temperature, incubated with monoclonal antibody 4A4 (1:400 dilution; Dako) at room temperature for 1 h, and subjected to signal development processes using the Vectastain Elite avidin-biotin peroxidase complex kit according to the manufacturer's protocol (Vector Laboratories). The slides were counterstained with Mayer's hematoxylin (DakoCytomation). When the slides were evaluated, nuclear immunoreactivity was considered, and ΔNp63 expression was scored, taking into account the intensity of nuclear staining (0-3), the thickness [from 0 (one third of the epithelial layer) to 3 (all the thickness of the epithelial layer)], and the gradient of ΔNp63 expression in the epithelial layer [from 0 (no expression) or 1 (conserved gradient of expression) to 3 (complete loss of the gradient of expression)]. The score was defined as the sum of these three criteria (0-9). Based on our previous work, a common feature in normal oral epithelium was an intensity of ΔNp63 staining of 1 to 2 in one third or two thirds of the epithelial layer with a conserved gradient of expression (score 0-5; ref. 20). Accordingly, OPLs with a score 0 to 5 were considered as negative, and OPLs with a score 6 to 9 were considered as positive.

We also evaluated podoplanin expression, considering cell membrane immunoreactivity; the expression was scored as negative (0-1) or positive (2-4) as previously reported (10).

During the evaluation of p63 immunostaining, we noticed that some samples exhibited clusters of intraepithelial inflammatory cells (EIC) in the basal layer of the epithelium. The presence of isolated EIC was a common feature, but clusters of EIC were clearly identified and defined by a cluster of at least 10 inflammatory cells in the thickness of the epithelium. The presence of inflammatory cells in the stroma was not considered in the scoring.

All the different scores were based on examination of the whole section in each biopsy under a multiheaded microscope by three observers (H.R., L.M., and A.K.E-N. for podoplanin and P.S., L.M., and A.K.E-N. for ΔNp63 and EIC), who were blinded to the clinical information.

Statistical analysis. The associations between podoplanin expression, ΔNp63 expression, EIC status, and clinicopathologic parameters were evaluated using the χ^2 test or Fisher's exact test for categorical variables and the Wilcoxon rank sum test for continuous variables. For time-to-event analysis, Kaplan-Meier curves were plotted. The median time to event with 95% confidence interval (95% CI) and event-free survival rates at years 3, 5, and 10 were determined. Cox proportional hazards models were used for unicovariate and multicovariate analyses. The hazard ratios (HR) with 95% CIs and *P* values were reported. All tests were two sided.

Results

Characteristics of the patients. Of the 162 patients enrolled in the chemoprevention trial, 10 (6%) had either lack of sufficient tissue in blocks or the disappearance of the squamous epithelial lesions. In 18 (12%) of the 152 patients with analyzable tissue in this study, biopsy specimens were obtained after enrollment because the paraffin blocks from baseline biopsies were unavailable. The medium follow-up period for the patient population was 7.5 years, with 36 (24%) of the 152 patients developing invasive cancer in the oral cavity: 18 tumors at the same sites of the original OPL and 18 tumors at different sites from the original OPL.

Expression of ΔNp63, EIC, and podoplanin in OPL. Expression of ΔNp63 was mainly nuclear in squamous epithelial cells. In contrast to the pattern of podoplanin expression (10), ΔNp63 immunostaining was homogeneous within a given lesion. ΔNp63 expression was observed in 144 (95%) of the 152 lesions; only 8 (5%) samples had no staining. In contrast, 28% of lesions had no podoplanin expression. Although nearly universal ΔNp63 nuclear staining was noted, the intensity of the expression and the extent of positivity in the epithelium varied considerably. The intensity of expression was scored as 1 in 18 (12%) cases, as 2 in 59 (39%) cases, and as 3 in 67 (44%) cases. The thickness of ΔNp63 expression in the epithelial layer was

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scored as 1 in 85 (56%) cases, as 2 in 51 (34%) cases, and as 3 in 8 (5%) cases. The gradient was scored as 1 (conserved) in 120 (79%) cases, as 2 (partial loss) in 13 (9%) cases, and as 3 (complete loss) in 11 (7%) cases. Among 152 evaluable cases, 111 (73%) cases were classified as negative (score 0-5) and 41 (27%) cases were classified as positive (score 6-9) for ΔNp63 (Fig. 1A and B).

While reviewing the expression of ΔNp63, we observed that some samples exhibited clusters of EIC in the basal layer of the epithelium. Clusters of EIC were clearly identified in 37 (26%) of 145 evaluable samples, usually in the basal layers (Fig. 1C and D).

Podoplanin expression in this cohort of patients with OPL has been previously reported (10). In summary, expression of podoplanin was highly variable and observed on the cell membrane, in pockets, predominantly at the basal layer. Of 150 evaluable OPL lesions, 56 (37%) were classified as podoplanin positive, and the remaining 63% were classified as podoplanin negative.

Correlation of ΔNp63, EIC, podoplanin, and clinicopathologic parameters. The distribution of the podoplanin expression status and its association with general clinicopathologic parameters are reported previously (10). In summary, podoplanin positivity was more frequent in older patients, female, and dysplastic lesions. The expression of ΔNp63 was more frequent in female ($P = 0.02$) and white ($P = 0.04$) populations. An association between ΔNp63 expression and histologic status was observed, without reaching statistical significance ($P = 0.06$). There was no statistically significant correlation between ΔNp63 expression status and smoking history or history of alcohol consumption. The presence of clusters of EIC was associated with older patients; the median ages were 63 years for patients with clusters of EIC and 53 years for patients without clusters of EIC ($P = 0.008$). The presence of clusters of EIC was not correlated with sex, race, histologic status, smoking history, or history of alcohol consumption (Table 1). A highly significant association was observed between ΔNp63 and podoplanin status ($P < 0.0001$), between ΔNp63 status and the presence of clusters of EIC ($P = 0.0003$), and between podoplanin status and the presence of EIC ($P = 0.0012$). Despite these findings, we observed that, among 41 OPL positive for ΔNp63, 12 (29%) were negative for podoplanin and that, among 111 OPL negative for ΔNp63, 25 (23%) were positive for podoplanin. The presence of all three positive biomarkers was associated with dysplastic histology ($P = 0.016$) and older age ($P = 0.004$).

Expression of ΔNp63, podoplanin, EIC, and oral cancer risk. In the unicovariate analysis, sex, race, age, smoking, and alcohol history were not significantly associated with oral cancer development. OPL histologic status was associated with oral cancer development but barely reached statistical significance ($P = 0.05$). Patients with podoplanin-positive OPL had a significantly higher incidence of oral cancer than did those with podoplanin-negative OPL ($P < 0.0001$, log-rank test; Table 1). Patients with ΔNp63-positive OPL had an increased oral cancer risk, particularly during the first 3 years

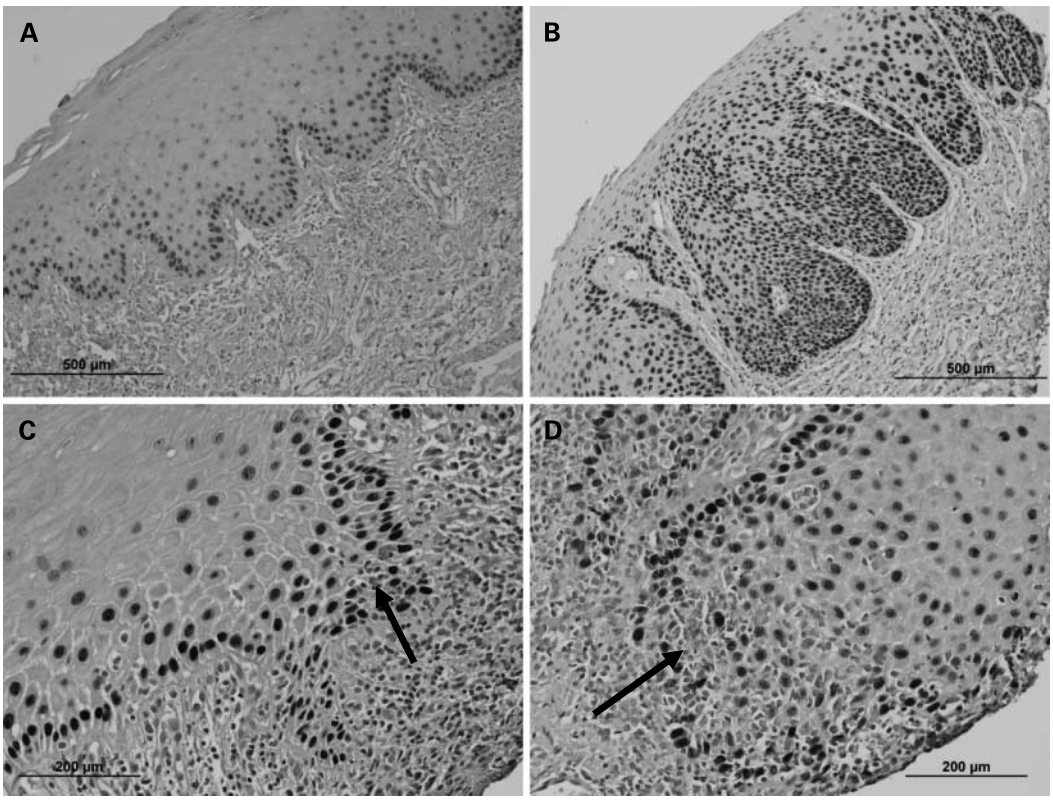

Fig. 1. ΔNp63 expression and EICs. *A,* a typical oral leukoplakia with negative ΔNp63 expression. *B,* a high-grade dysplasia with positive ΔNp63 expression. *C* and *D,* two OPLs showing clusters of EIC (*arrows*).

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Table 1. Median time to oral cancer development (in years) and the OCFS rates at years 5 and 10, along with the 95% CIs for all patients and for each group

Variable	*n*	Oral cancer	Median (95% CI)	OCFS rate at 5 y (95% CI)	OCFS rate at 10 y (95% CI)	*P*
All patients	162	39	14.34 (NA)	0.8 (0.74-0.87)	0.73 (0.66-0.81)	
Sex						0.80
Female	77	18	NA	0.78 (0.69-0.88)	0.73 (0.63-0.85)	
Male	85	21	14.34 (NA)	0.82 (0.74-0.91)	0.73 (0.63-0.85)	
Race						0.74
Other	17	3	NA	0.85 (0.67-1)	0.75 (0.54-1)	
White	145	36	14.34 (NA)	0.8 (0.73-0.87)	0.73 (0.66-0.82)	
Histologic status						0.05
Hyperplasia	109	22	14.34 (NA)	0.84 (0.78-0.92)	0.79 (0.71-0.88)	
Mild dysplasia	40	11	NA	0.75 (0.63-0.91)	0.67 (0.53-0.86)	
Moderate/severe dysplasia	13	6	6.67 (1.85-NA)	0.59 (0.37-0.95)	0.47 (0.25-0.9)	
Treatment arm						0.66
13cRA	81	20	NA	0.78 (0.69-0.88)	0.72 (0.62-0.83)	
BC + RP	45	10	14.34 (NA)	0.84 (0.73-0.96)	0.8 (0.69-0.94)	
RP	36	9	NA (6.65-NA)	0.82 (0.69-0.96)	—	
Smoking history						0.26
Current	56	8	NA	0.86 (0.77-0.96)	0.83 (0.72-0.95)	
Former	65	21	14.34 (10.7-NA)	0.78 (0.68-0.89)	0.67 (0.55-0.81)	
Never	41	10	NA	0.77 (0.65-0.91)	0.74 (0.61-0.89)	
Alcohol history						0.74
Current	93	21	14.34 (NA)	0.84 (0.76-0.92)	0.75 (0.66-0.86)	
Former	19	5	NA (6.21-NA)	0.78 (0.61-1)	0.68 (0.48-0.98)	
Never	50	13	NA	0.75 (0.64-0.88)	0.72 (0.6-0.87)	
Podoplanin expression						<0.0001
0, 1	94	13	14.34 (NA)	0.93 (0.87-0.99)	0.83 (0.74-0.92)	
2, 3, 4	56	22	10.7 (6.67-NA)	0.63 (0.51-0.77)	0.6 (0.47-0.75)	
ΔNp63 expression						<0.0001
0-5	111	17	14.34 (NA)	0.89 (0.83-0.95)	0.81 (0.73-0.9)	
6-9	41	19	10.7 (3.52-NA)	0.61 (0.47-0.78)	0.54 (0.4-0.73)	
Clusters of EIC						0.015
Absent	108	18	NA	0.86 (0.79-0.93)	0.79 (0.71-0.89)	
Present	37	14	10.7 (6.67-NA)	0.72 (0.59-0.89)	0.62 (0.47-0.81)	
No. positive biomarkers						<0.0001
0, 1, 2	140	26	14.34 (NA)	0.85 (0.79-0.92)	0.78 (0.71-0.87)	
3	15	11	2.46 (1.62-NA)	0.39 (0.2-0.74)	0.31 (0.14-0.68)	

NOTE: *P* values from the log-rank test (unicovariate analysis) are also provided.

of follow-up ($P < 0.0001$, log-rank test; Fig. 2). At 5 years after the randomization date, the oral cancer–free survival (OCFS) rate for the patients with ΔNp63-negative OPL was 89% (95% CI, 0.83-0.95) compared with 61% for the patients with ΔNp63-positive OPL (95% CI, 0.47-0.78; Table 1). The risk of developing oral cancer was less marked when considering the presence of clusters of EIC but was statistically significant ($P = 0.015$, log-rank test; Fig. 2). At 5 years after the randomization date, the OCFS rate for the patients with no clusters of EIC was 86% (95% CI, 0.79-0.93) compared with 72% for the patients with clusters of EIC (95% CI, 0.59-0.89; Table 1).

We then looked at the probability of developing oral cancer considering all three biomarkers. When all three biomarkers were positive, the OCFS was strikingly lower; at 5 years after randomization, the OCFS rate for the patients with three positive biomarkers was 39% (95% CI, 0.20-0.74) compared with 85% (95% CI, 0.79-0.92) for the patients with no, one, or two positive biomarkers ($P < 0.0001$; Table 1). When using Bonferroni method to adjust for multiple comparisons, the differences in time to oral cancer for podoplanin, ΔNp63, and all three markers combined remain significant when comparing with the significance level of 0.0125. However, the difference in time to oral cancer for clusters of EIC becomes marginally significant.

For multicovariate analysis, the Cox proportional hazards model was fitted for the three biomarkers and the group of biomarkers separately because they were highly associated with each other (Table 2). For each model, age, treatment arm, and histologic status and a specific biomarker or group of biomarkers were entered. In model 1 (Table 2A), podoplanin was the only independent factor, with a HR of 3.094 (95% CI, 1.505-6.363; $P = 0.002$). In model 2 (Table 2B), ΔNp63 expression was an independent factor with a HR of 3.308 (95% CI, 1.663-6.580; $P = 0.0007$), whereas age was another independent factor but less significant ($P = 0.02$). In model 3 (Table 2C), age and histologic status were the only independent factors; the presence of clusters of EIC did not reach statistical significance. In model 4 (Table 2D), all three biomarkers being positive was the only independent factor for cancer development and was associated with the highest HR of 4.372 (95% CI, 1.912-9.992; $P = 0.0005$).

Among 36 patients who developed oral cancer and for whom biomarker data were available, podoplanin and ΔNp63 were coexpressed in 16 (45%) cases, neither of them was positive

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in 12 cases (33%), and interestingly, only one of them was expressed in 8 (22%) cases. One of the 12 patients with negative podoplanin and ΔNp63 expression exhibited clusters of EIC. Among 36 patients who developed oral cancer and for whom ΔNp63 data were available, 79% of the patients who were positive for ΔNp63 developed oral cancer at 3 years of follow-up compared with 47% of the patients who were negative for ΔNp63.

Among all cases studied, the cumulative incidence rate for developing oral cancer at 3 years of follow-up was 53% for the patients with all three positive biomarkers compared with 11% of the other patients. When we considered only oral cancers that developed in the same site as the OPL, 25% of the patients positive for podoplanin developed cancer compared with 4% of the patients negative for podoplanin, 24% of the patients positive for ΔNp63 developed cancer compared with 7% of the patients negative for ΔNp63, and 40% of the patients positive for all the biomarkers developed oral cancer compared with 9% of the patients with no, one, or two positive biomarkers.

Discussion

In this study of a large series of patients included in a prospective chemoprevention trial with a median follow-up of 7.5 years, we show for the first time the value of ΔNp63 expression as a predictor of oral cancer development. In a multicovariate analysis that included age, treatment arm, and histologic status, ΔNp63 expression was independently associated with oral cancer development with a HR of 3.308 (95% CI, 1.663-6.580). At 5 years after the randomization date, 39% of the patients with positive ΔNp63 staining had developed cancer compared with only 11% of patients with negative ΔNp63 staining. Interestingly, we observed that the presence of clusters of EIC was associated with an increased risk of developing oral cancer, although this risk was marginally significant in the multicovariate analysis. The significance of ΔNp63 expression in predicting oral cancer development was comparable with that of podoplanin. Because these two factors were highly associated, analyzing them together in a multicovariate analysis was difficult. Nevertheless, when OPLs were positive for all three risk biomarkers, patients had the greatest risk of developing oral cancer, 61% at 5 years with a HR of 4.372 (95% CI, 1.912-9.992).

The samples used in our study were collected in the largest and longest-term prospective, randomized trial conducted in OPL patients to date and the first to include a prespecified secondary analysis of long-term oral cancer incidence (19).

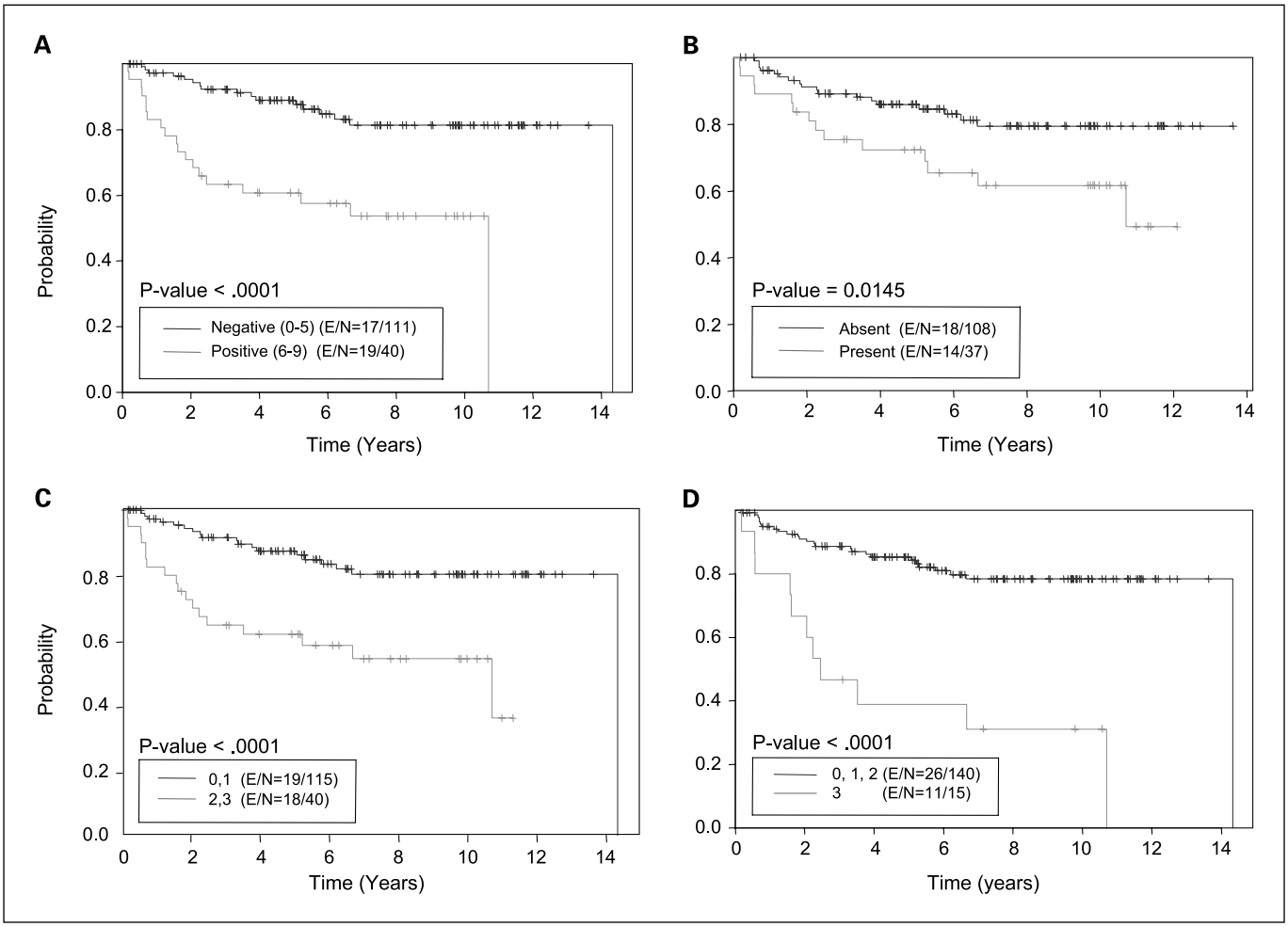

Fig. 2. OCFS by patient prognostic factors: ΔNp63 expression (*A*), presence of EICs (*B*), and combinations of biomarkers: 0 or 1 versus 2 or 3 (*C*) and 0, 1, or 2 versus 3 positive biomarkers (*D*). E/N, number of events and number of patients.

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Table 2. Multicovariate analysis of podoplanin, ΔNp63, clusters of EIC, and all three biomarkers for oral cancer development

Analysis of maximum likelihood estimates		
Variable	***P***	**HR (95% CI)**
A. Model 1 (E/N = 35/150)		
Age	0.05	1.031 (1.000-1.064)
BC + RP vs 13cRA	0.79	0.889 (0.378-2.090)
RP only vs 13cRA	0.41	1.426 (0.617-3.297)
Histologic status at baseline: dysplasia vs hyperplasia	0.11	1.776 (0.881-3.581)
Podoplanin: 2-4 vs 0-1	0.002	3.094 (1.505-6.363)
B. Model 2 (E/N = 36/152)		
Age	0.02	1.035 (1.004-1.066)
BC + RP vs 13cRA	0.95	1.027 (0.452-2.336)
RP only vs 13cRA	0.52	1.311 (0.576-2.981)
Histologic status at baseline: dysplasia vs hyperplasia	0.16	1.632 (0.827-3.222)
ΔNp63: 6-9 vs 0-5	0.0007	3.308 (1.663-6.580)
C. Model 3 (E/N = 32/145)		
Age	0.006	1.047 (1.013-1.082)
BC + RP vs 13cRA	0.49	0.727 (0.294-1.795)
RP only vs 13cRA	0.24	1.647 (0.714-3.796)
Histologic status at baseline: dysplasia vs hyperplasia	0.046	2.054 (1.013-4.164)
Clusters of EIC: 1 vs 0	0.057	2.032 (0.980-4.213)
D. Model 4 (E/N = 37/155)		
Age	0.12	1.024 (0.994-1.056)
BC + RP vs 13cRA	0.86	0.929 (0.413-2.093)
RP only vs 13cRA	0.19	1.757 (0.759-4.070)
Histologic status at baseline: dysplasia vs hyperplasia	0.36	1.385 (0.689-2.783)
No. positive biomarkers: 3 vs 0-2	0.0005	4.372 (1.912-9.992)

NOTE: For multicovariate analysis, the Cox proportional model was fitted for the three markers separately because they are highly associated with each other. For each model, age, treatment arm, and histologic status and a specific biomarker or group of biomarkers were entered.
Abbreviation: E/N, number of events and number of patients.

The results of this trial that compared low doses of 13cRA with RP with or without BC in OPL show similar OCFS rates across all arms. No significant association between clinical response at 3 months and longer OCFS was observed. Histologic responses were similar between all arms of the study. The conclusion of the trial was that 13cRA versus BC and RP or RP alone was ineffective in preventing oral cancer development. Our knowledge of the natural history of OPL is mainly based on a study that included 257 patients with untreated oral leukoplakia followed for an average period of 7.2 years (3). Of the initial biopsies, 235 (91%) revealed a benign hyperkeratosis and 22 (9%) others contained some degree of epithelial dysplasia. A total of 53 (21%) patients developed oral cancer. With a similar follow-up, we observed the same rate of transformation. This series of longitudinal and prospectively collected samples is therefore unique and represents an opportunity to identify biomarkers associated with a high risk to develop oral cancer (21).

Among the two major isoforms described for p63 (11), ΔNp63 variants are the predominant forms in epithelial cells. In our study, we use a monoclonal antibody raised against the NH_2 terminus of ΔNp63 (clone 4A4; ref. 13). In particular, ΔNp63 has been reported as the predominant form in epithelial cells and oral epithelial dysplasia and has been found to be overexpressed in HNSCCs (22–24). We previously reported that ΔNp63 expression was gradually increasing in extent and intensity with histologic progression of dysplasia. In carcinomas, p63 was almost always expressed (94.7%; ref. 20). These results and our present article are in line with p63 as an early event in HNSCC tumorigenesis.

Several mechanisms have been mentioned to explain the role of ΔNp63 as a promoter of tumorigenesis. In HNSCC, ΔNp63 up-regulates expression of keratins 6A and 14 and down-regulates expression of keratins 4 and 19, in keeping with their expression patterns in HNSCC (25). It has also been shown that ΔNp63 promotes survival in HNSCC (26) and is a positive regulator of the β-catenin signaling pathway (27). Finally, silencing of overexpressed ΔNp63 is associated with an inhibition of proliferation concurrently with an up-regulation of cyclin-dependent kinase inhibitors $p27^{kip1}$ and $p57^{kip2}$ (28).

We have shown a high podoplanin expression in 57% of squamous cell carcinoma of the oral cavity and its association with lymph node metastasis and a short disease-specific survival (29). Epidermal growth factor, basic fibroblast growth factor, and tumor necrosis factor have been shown to induce podoplanin expression, and podoplanin has been associated with tumor cell invasion in the absence of epithelial-mesenchymal transition pathway (30, 31). The underlying molecular players and mechanisms warrant further investigation.

The mechanism of the high association between ΔNp63 and podoplanin expression observed in our study is unclear. ΔNp63 is widely expressed in the basal layer but not in terminally differentiated squamous cells, suggesting a critical role of ΔNp63 in epithelial stem cell biology (11, 32). It has been recently shown that ΔNp63 acts on the CD44 protein in a fashion opposite to that of p53, by stimulating CD44 expression (33), and that a $CD44^+$ population of human HNSCCs possessed properties of cancer stem cells (34). Podoplanin expression was not detectable in normal oral epithelium or at the basal layer in most of the OPLs but extended to the suprabasal layer or above in some of the lesions. Similar to that of ΔNp63, podoplanin expression is mainly detected in squamous cell carcinomas and is rare in adenocarcinomas (31). Several lines of evidence suggest that both ΔNp63 and podoplanin are induced during tissue development and regeneration (11, 31). In an analysis of ME180 cervical carcinoma cells depleted of ΔNp63 expression, *podoplanin* was one of the most significantly down-regulated genes (35). However, in contrast to the nuclear localization of ΔNp63, podoplanin localizes on the cell membrane and is not always coexpressed in the same cells. Therefore, more studies are needed to determine the relationship between ΔNp63 and podoplanin.

The association between clusters of EIC and an increased risk of oral cancer development is interesting, although not very strong. An increase of immune cell infiltration has been associated with progression of oral epithelium from hyperkeratosis to dysplasia and carcinoma (36). A recent study by Katou et al. (37) showed that intraepithelial lymphocytes were immunosuppressive, whereas stromal lymphocytes were immunocompetent. However, oral cancers with $CD4^+$ lymphocytes infiltrating were associated with a favorable prognosis (38). Therefore, it will be important to better characterize inflammatory cells forming the

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clusters of EIC, as a specific population is perhaps associated with a higher risk of developing oral cancer. Because human papillomavirus has been implicated in oral cancer development (39), it will be also important to determine its association with clusters of EIC.

BC is a precursor of vitamin A, RP is a form of vitamin A, and 13cRA is derived from vitamin A. Vitamin A has been shown to have an unexpected and important effect on the immune response (40). However, no significant association was observed between the presence of clusters of EIC and response to treatment (data not shown).

We previously reported that more podoplanin-positive OPLs were observed in the group that developed cancer at the same site than in the group that developed cancer at different sites, even if the difference was not statistically significant (10). This was not the case for ΔNp63 and clusters of EIC where the distribution of positive samples was the same in the group that developed cancer at the same site and in the group that developed cancer at different sites (data not shown). This observation raises the possibility that ΔNp63 and clusters of EIC may reflect field cancerization phenomena, and podoplanin a more focal and clonal expansion.

Previous studies showed that loss of heterozygosity at 3p and/or 9p in patients with OPL was associated with a 25% to 40% 3-year cancer risk (41–43). This parameter is being used to select high-risk patients in an ongoing phase III oral cancer prevention trial targeting epidermal growth factor receptor (9). Clusters of EIC, podoplanin, and ΔNp63 expression had similar predictive value (25-38% 3-year cancer risk; data not shown), whereas triple-positive patients had a 53% 3-year cancer risk. Because the measurement of the three biomarkers can be done in routine pathology laboratories, in practice, it may have an advantage over the more sophisticated genetic analysis (44).

One of the important issues in biomarker development is whether the biomarkers can be used to guide treatment. In basal-like breast cancers, both epidermal growth factor receptor and ΔNp63 are often coexpressed (45). In our future studies, it will be interesting to test whether there is a correlation between the two factors in OPL because epidermal growth factor receptor is a valid therapeutic target.

Taken together, the high oral cancer predictive value using the biomarkers reported in this study is promising and warrants further validation in other prospective longitudinal cohort studies, preferably in general populations to avoid the potential patient selection bias inherent in most therapeutic/prevention trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ΔNp63 Overexpression, Alone and in Combination with Other Biomarkers, Predicts the Development of Oral Cancer in Patients with Leukoplakia

Pierre Saintigny, Adel K. El-Naggar, Vali Papadimitrakopoulou, et al.

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