Azienda Ospedaliera S. Croce e Carle Cuneo

Prognostic value of HPV detection with three primer sets in 255 Head-Neck cancers

no.6045

Cristiana Lo Nigro, PhD,[1a*] Daniela Vivenza, PhD,[1a] Martino Monteverde, PhD,[1a] Mirella Fortunato, MD,[2] Nerina Denaro, MD,[1b] Laura Lattanzio, PhD,[1a] Federica Tonissi, M.Sc.,[1a] Valentina Astesana, M.Sc.,[1a] Elvio Russi, MD,[3] Marco C. Merlano, MD,[1b]

[1a]Laboratory of Cancer Genetics and Translational Oncology and [1b]Medical Oncology, Oncology Department, S. Croce Teaching Hospital, via Carle 25, 12100, Cuneo, Italy; [2]Pathology Department, S. Croce Teaching Hospital, via Coppino 26, 12100, Cuneo, Italy; [3]Radiotherapy Department, S. Croce Teaching Hospital, via Coppino 26, 12100, Cuneo, Italy.

2015 ASCO Annual Meeting Illumination & Innovation transforming data into learning May 29-June 2, 2015 McCormick Place | Chicago, Illinois ASCO

BACKGROUND

Head and neck cancer (HNC) represents the sixth most common cancer worldwide [1]. Tobacco and alcohol abuses remain the strongest established risk factors, but accumulating data indicate that oncogenic HPVs are associated with squamous cell lesions of HN [2]. Oropharynx is the favourite HPV infection site where the most HN HPV-related tumours arise, and it is worldwide accepted that HPV-positive patients show a better prognosis than HPV-negative ones, with the depiction of a distinct tumour entity [3]. This strong relationship between HPV and Oropharynx Cancers (OPCs) is not so evident in the other HN Squamous Cell Carcinoma (SCC) primary sites [4]. The persistent infections and the viral genome integration represent the prerequisite for the development of invasive cancers, in which the viral proteins E6 and E7 play a key role in malignant transformation [5]. Integration leads usually to disruption and deletion of some viral genes such as L1, coding for the major capsid protein, and E1 and E2 which are of importance in viral replication and transcription [6].

To date, PCR represents the most feasible, sensitive, available and cost-effective method for HPV detection but shows a low capability in distinguishing clinically relevant HPV infections. In this sense, mRNA quantification of E6/E7 in frozen tissues is considered the gold-standard test for relevant infections, but it does not seem generally feasible on formalin-fixed paraffin-embedded (FFPE) archival tissue samples [7].

In clinical trials the combination of immunohistochemical staining (IHC) for p16 expression together with in situ hybridization (ISH) for HPV detection is the standard procedure for the identification of HPV-related HNC, since each method is not sufficient for an adequate specificity and sensitivity [8].

Therefore a single and adequate marker of HPV-induced HNSCC is still lacking.

AIM

In this study we investigated the prevalence of HPV infection by DNA-PCR in a series of locally advanced SCC of the head and neck (LA-HNC) and compared the prognostic value of E1, E6 and L1 genomic viral fragments, each other in order to find the best prognosticator among them in terms of Overall Survival (OS) and Progression Free Survival (PFS). Moreover, we evaluated these viral markers in non-OPCs, to establish also a possible role of HPV outside OPCs.

PATIENTS AND METHODS

We have retrospectively collected a series of 255 histological confirmed patients with LA-HNC stage III-IV, treated with curative intent by chemo-radiation (CRT) between 1997 and 2013 at the S. Croce Teaching Hospital, Cuneo, Italy. An informed consent for scientific purpose was obtained from each patient enrolled in the study that was conducted in respect to Helsinki Declaration.

DNA extraction and HPV16 detection

DNA samples were extracted from FFPE tissues at diagnosis, by a standard protocol that included proteinase K treatment.

Genomic viral fragments from E1, E6 and L1 genes were detected by qualitative PCR, using specific primer pairs for HPV type 16, as it follows:

E6 forward 5' -CGGTTGAACCGAAACCGG-3';
E6 reverse 5' -CCTGTGGGTCCTGAAACATT-3';
L1 forward 5' -GCACAGGGCCACAATAATGG-3';
L1 reverse 5' -TGGCAGCACATAATGACATATT-3';
E1 forward 5' -GAGATGCAGTACAGGTTCTAAAACG-3';
E1 reverse 5' -TGCCATACCCGCTGTCTTC-3'.

DNAs from CaSki cell line and human lymphocyte of healthy donors, were used as pos and neg controls respectively

Each patient was analysed simultaneously for the 3 viral primer sets and for a housekeeping gene such as GAPDH in order to determinate the integrity of tumour DNA samples.

Primers used for GAPDH amplification were as follows:

forward 5'-TCACCAGGGCTGCTTTTAAC-3'
reverse 5'-GGCTCACCATGTAGCACTCA-3'.

Amplicons were visualized on 2% agarose gel under UV light.

Patients with scanty DNA samples were rejected and excluded from analyses.

Statistical Methods

HPV16 prevalence was based on the detection of at least one viral fragment in tumour tissue; then it was calculated for each viral fragment: E1, L1 and E6.

OS analyses were based on the time from treatment start to death or last contact in which the survivors were censored. PFS analyses were based on the time from treatment start to first event; patients without an event were censored at their last follow-up. OS was calculated using the Kaplan-Meier method with log-rank test for statistical significance.

Relationships between HPV status and clinical parameters (gender, age, smoke and T, G, N) were analysed by cross-tabulation. Unadjusted associations between categorical were tested with Pearson's test Mann-Whitney test was used for assessing difference between genders for tobacco consumption.

Statistical significance was established at the 5% level (P<0.05) for all statistical analysis.

The analyses were performed using the statistical GraphPad Prism 5 (San Diego, CA, US) and SPSS version 13 (SPSS, Chicago, IL) programs.

RESULTS

Demographic and clinical pathological characteristics

Tumour tissue specimens from 255 of LA-HNC patients were available for the study. They were of Caucasian origin and showed a median follow-up of 25 months (range 1-206). At treatment start the age of patients ranged from 21 to 86 years with a median of 61 years. Two hundred and fifteen (84.3%) of the patients were male and 40 (15.7%) were females. Eighty-nine (34.9%) LA-HNC arose in the oropharynx and 166 (65%) in non-oropharynx; which in turn included 70 hypopharynx (27.4%), 51 larynx (20%), 42 oral cavity (16.5%) and 3 nasopharynx (1.2%) tumours.

The distributions of gender, primary sites, performance status (PS), tumor size (T), nodal status (N), histological grade (G) and smoking habitude are reported in **Table 1**.

Table 1. Characteristics of LA-HNC pts (N=255) and tumours.

	Number of patients	Rates	
Gender			**Median age (range) yrs**
Male (M)	215	84,3%	61 (36-86)
Female (F)	40	15,7%	62 (21-83)
Primary Sites			
Oropharynx	89	34,9%	
Hypopharynx	70	27,4%	
Larynx	51	20,0%	
Oral Cavity	42	16,5%	
Nasopharynx	3	1,2%	
Performance Status			
PS0	130	51,0%	
PS1	102	40,0%	
PS2	7	2,7%	
NA	16	6,3%	
Tumour size			
T1/T2	81	31,8%	
T3/T4	169	66,2%	
NA	5	2,0%	
Nodal Status			
N0	25	9,8%	
N1	25	9,8%	
N2/N3	201	78,8%	
NA	4	1,6%	
Grade			
G1/G2	115	45,1%	
G3	104	40,8%	
NA	36	14,1%	
Smoke			
Heavy Smokers	185	72,6%	
Non Smokers	21	8,2%	
Unknown	49	19,2%	
Year of diagnosis			
1997-2005	84	33,0%	
2006-2013	171	67,0%	

NA = not available.

No significant differences were observed in the baseline clinical and pathological characteristics, as PS, T, G and smoke among primary tumour sites, **while** an increasing % of nodal involvement lymph nodes **was** observed in larynx tumours (**N0**=29.41%) compared to other sites (P<0.0001). The prevalence of women in oropharynx and oral cavity tumours was significantly higher than in larynx and hypopharynx primary sites (P=0.004), **but in the** retrospective data on smoking men were both more frequent (76.7% vs 50%, P<0.0001) and heavier smokers (median of 35 packs/year vs 28 packs/year, P=0.012) than women.

HPV prevalence in LA-HNC patients and association with tumour characteristics

Overall, 136 of 255 patients (53.3%) were HPV16 positive with at least one of the primer pairs used.

Among them an increasing number of positive samples was found, from E1 (35/255; 13.7%) to L1 (76/255; 29.8%) and E6 (130/255; 51%).

Therefore each pos patient highlighted variable positivity for E1, L1 and E6 viral fragments. Interestingly, E1 detection was always linked to E6 and L1 positivity.

In this sense, inside the HPV16 pos group of 136 patients we identified 2 subgroups with decreasing viral positivity:

35 patients (25.7%) were simultaneously pos for the 3 viral fragments (E1+L1+E6+), while 101 (74.3%) patients were pos only for 2 (L1+E6+) or at least 1 viral fragment (L1+E6- or L1-E6+) and never for E1 (E1-and/orL1+E6+).

One hundred nineteen patients were resulted HPV16-neg by all primer pairs used.

HPV prevalence in OPC and non-OPC patients and association with tumour characteristics

Tumours were grouped into OPC and non-OPC patients.

Overall, OPCs showed a higher HPV16 prevalence (64/89; 72%) compared to non-OPCs (72/166; 43.4%) (P<0.0001), moreover the pos sample proportions of for each fragment analysed were kept in both groups, with the highest % for E6 and the lowest for E1 (**Table 2**).

Table 2. Comparison of the power detection among E1, E6 and L1 viral fragments in OPCs and non-OPCs. The prevalence of pos samples, for each viral fragment analyzed is higher in OPC group than in the non-OPC one. The % of pos E1 patients was significantly lower both in OPC and non-OPC compared with pos L1 and E6 patients.

Whole cohort of LA-NHC patients

	Overall prevalence	Prevalence of each primer sets						
Number of patients analysed by PCR	pos samples	E1 Pos (%)	E1 Neg (%)	L1 Pos (%)	L1 Neg (%)	E6 Pos (%)	E6 Neg (%)	P value (b)
Total OPCs and non-OPCs (N=255 and %)	136 (53.3%)	35 (13.7%)	220 (86.3%)	76 (29.8%)	179 (70.2%)	130 (51 %)	125 (49%)	E1 vs L1 P <0.0001* E1 vs E6 P <0.0001* L1 vs E6 P <0.0001*

OPC and non-OPC patients

	Overall prevalence	Prevalence of each primer sets						
Number of patients analysed by PCR	pos samples	E1 Pos (%)	E1 Neg (%)	L1 Pos (%)	L1 Neg (%)	E6 Pos (%)	E6 Neg (%)	P value (b)
OPCs (N=89 and %)	64 (72%)	28 (31.5%)	61 (68.5%)	48 (53.8%)	41 (46.1%)	60 (67.4%)	29 (32.6%)	E1 vs L1 P=0.0024* E1 vs E6 P <0.0001* L1 vs E6 P=0.066
Non-OPCs (N=166 and %)	72 (43.4%)	7 (4.2%)	159 (95.8%)	28 (16.9%)	138 (83.1%)	70 (42.2%)	96 (57.8%)	E1 vs L1 P= 0.0002* E1 vs E6 P <0.0001* L1 vs E6 P<0.0001*
P value (a)	P<0.0001	P<0.0001*		P<0.0001*		P=0.000121*		

P value = P was obtained by Pearson's Test.

(a) = The results were obtained analysing OPCs vs non-OPCs for each viral fragments

(b) = The results were obtained analysing E1 vs L1 vs E6 and vs p16 status.

* = significant result

OPC patients showed also higher % of E1+L1+E6+ tumours (28/89; 31.5%) and lower % of HPV16-neg ones (25/89; 28.1%) compared to non-OPCs (7/166; 4.2% for E1+L1+E6 and 94/166; 56.7% for HPV neg patients) (P<0.0001), while there was no difference in the % of E1-and/orL1+E6+ tumours in both groups (36/89; 40.4% for OPCs and 65/166; 39.1% for non-OPCs; P=0.84) (**Figure 1**).

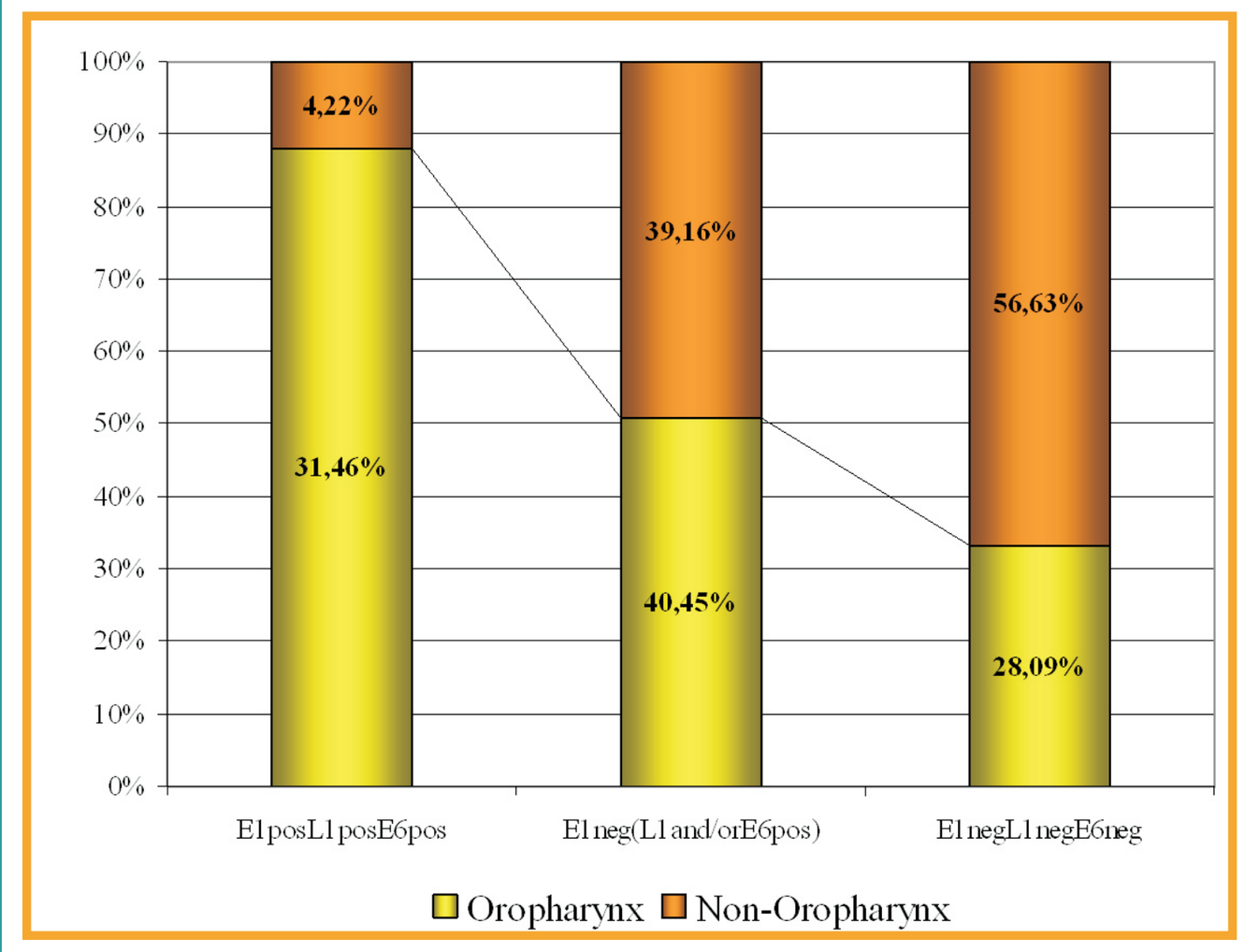

Figure 1: Distribution of positive fragments in LA-HNC sites. Histograms show a higher rate of positive samples for each fragment analyzed (E1+L1+E6+) in OPCs (31.46%) compared with non-OPCs (4.22%).

Positivity distributions in OPCs and in non-OPCs site by site are depicted in **Figure 2**.

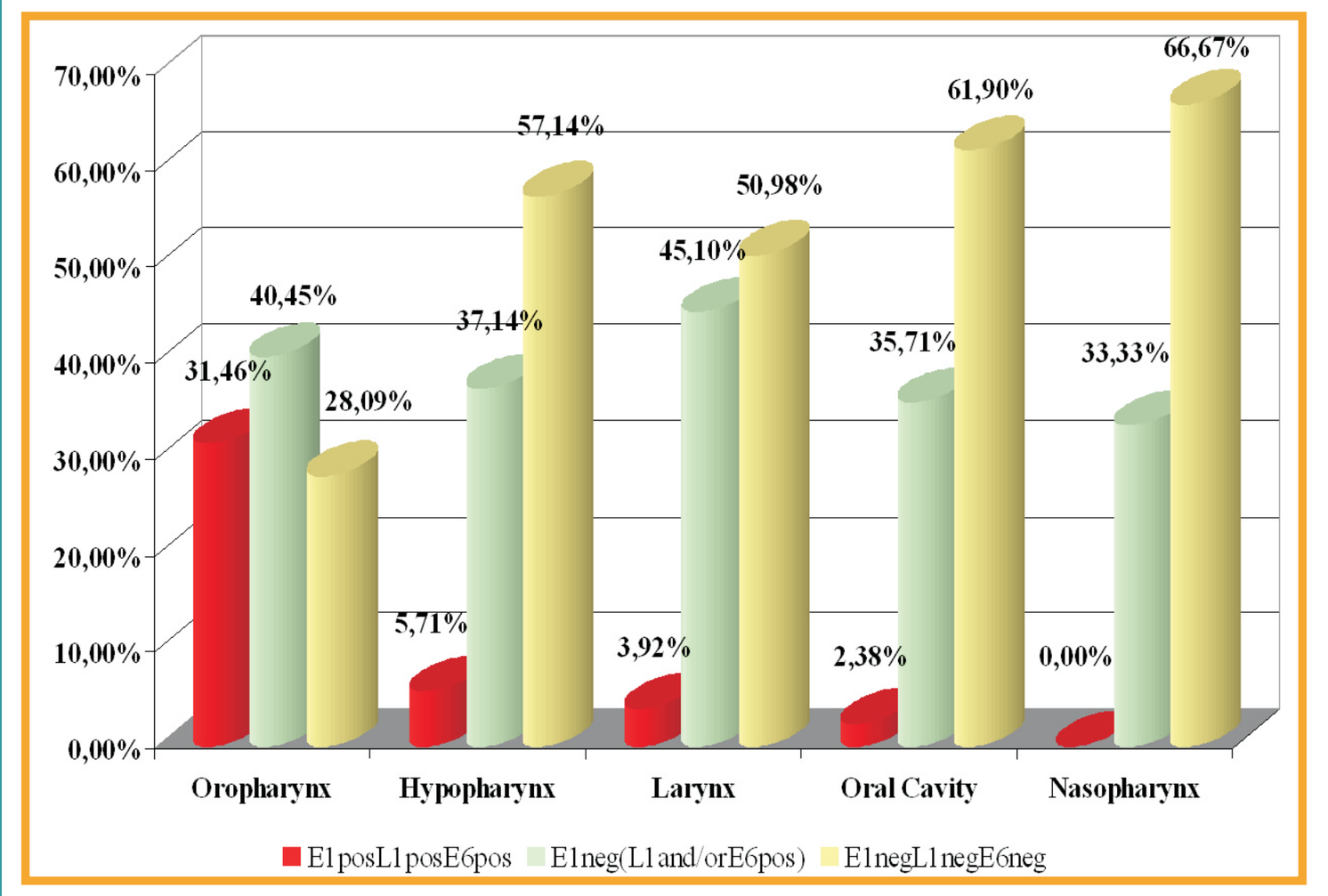

Figure 2: Distribution of positive and negative fragments according to combinations of viral status for each fragment in OPC and non-OPC patients by primary site.

Survival analysis in OPC patients

To determine whether the presence of HPV found with various degrees of positivity had prognostic significance we analysed both OS and PFS.

In term of prognostic value we compared the patients with different combined viral positivity and the HPV16-neg ones.

The E1+L1+E6 OPC patients had significantly improved OS (P=0.037) and PFS (P=0.056) with unreached the median for both, while E1-and/orL1+E6+ patients had the worst (median 20 and 27 months for OS and PFS respectively). Kaplan-Meier survival analysis showed that HPV16-neg patients demonstrated both lower OS and PFS compared to E1+L1+E6+ ones, but longer than E1-and/orL1+E6+ (**Figure 3**).

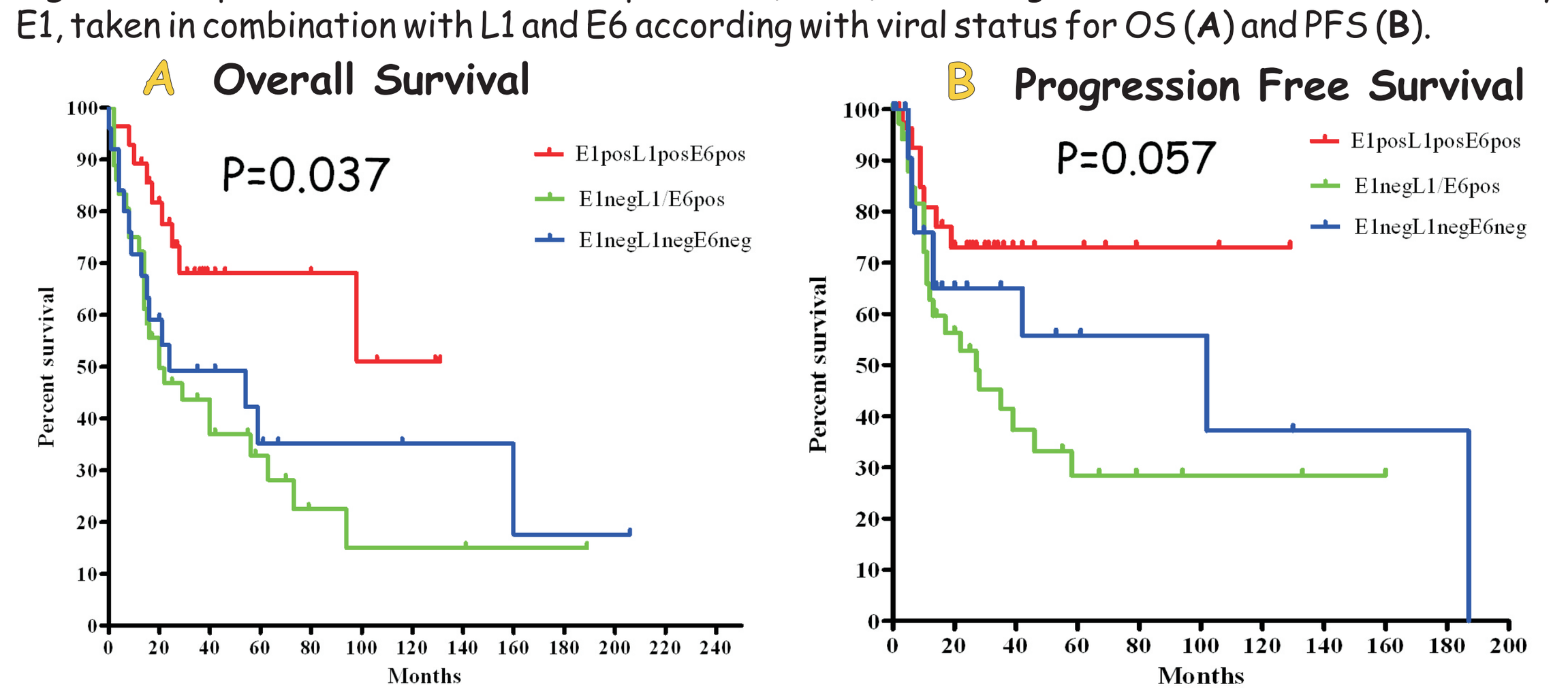

Figure 3. Kaplan-Meier curves in OPC patients (N=89) according to tumor HPV status defined by E1, taken in combination with L1 and E6 according with viral status for OS (**A**) and PFS (**B**).

It's evident that being E1 detection always associated with L1 and E6, E1 positivity alone might be sufficient to correlate with increased OS (P=0.012) and PFS (P=0.036) (**Figure 4**).

On the contrary, when L1 and E6 fragments were assessed for Kaplan-Meier analysis, we found that pos patients for both fragments had higher OS than the neg ones, but neither L1 nor E6 individually were associated with any gain in survival (**Figure 5**).

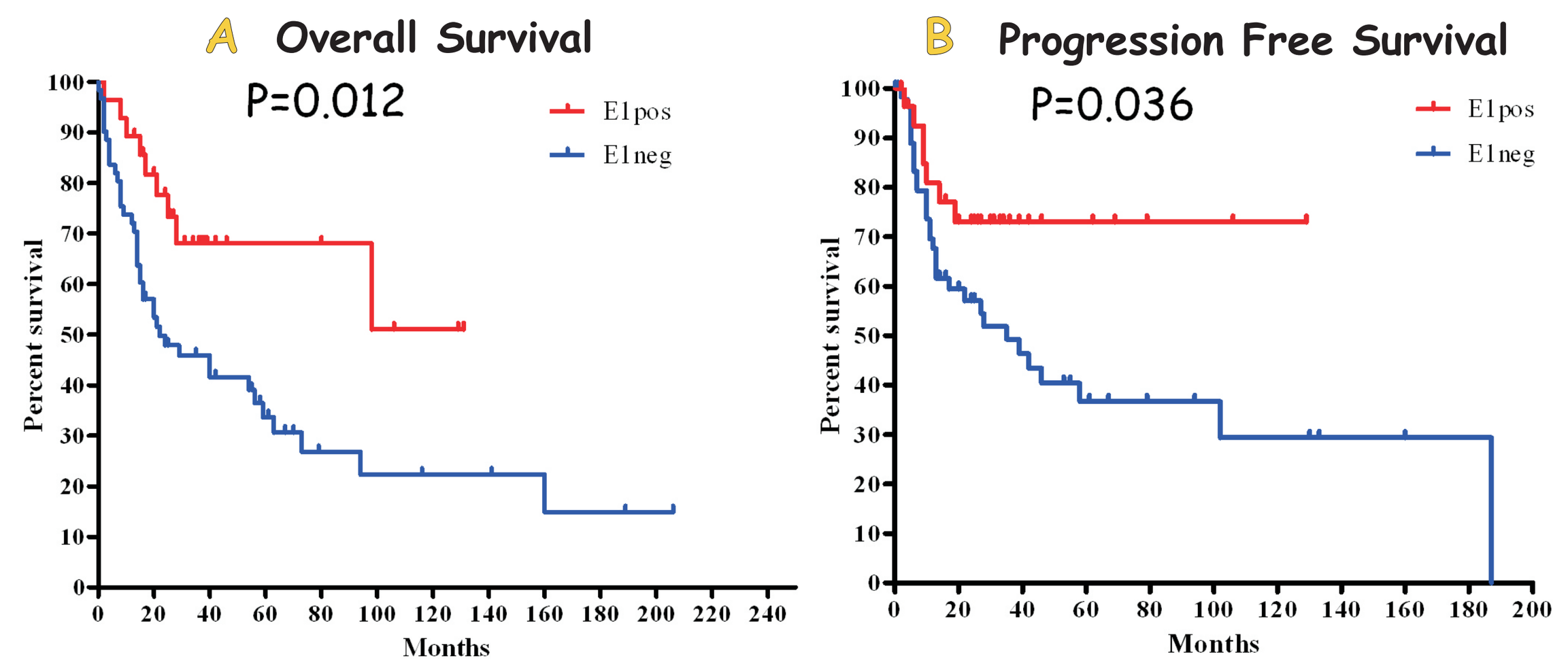

Figure 4. Kaplan-Meier curves in OPC patients (N=89) according to tumor HPV status defined by E1 for OS (**A**) and PFS (**B**).

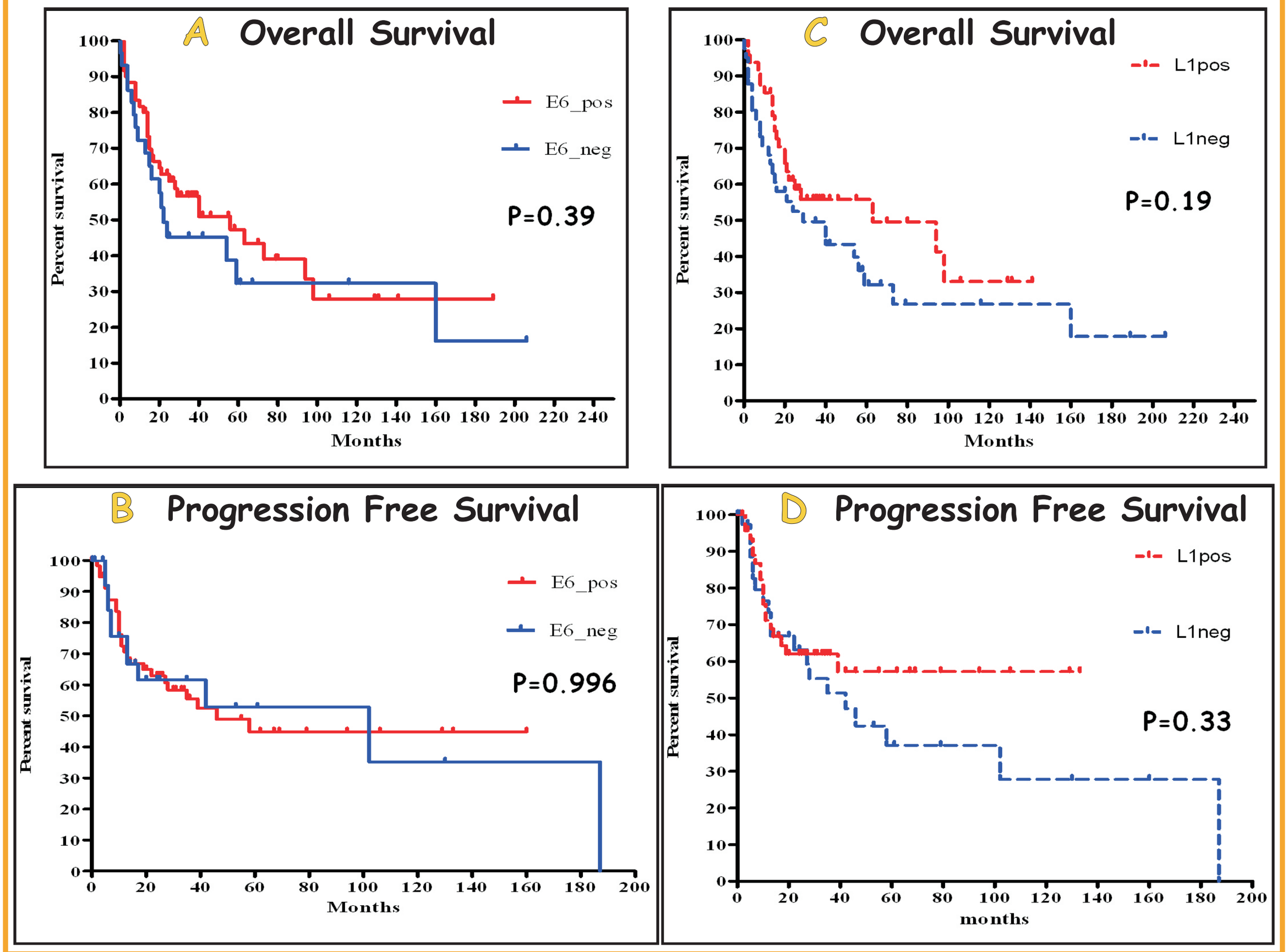

Figure 5. Kaplan-Meier curves in OPC patients (N=89) according to tumor HPV status defined by E6, for OS (**A**) and PFS (**B**) and by L1 for OS (**C**) and PFS (**D**), taken individually.

Survival analysis in non-OPC patients

In non-OPC patients we detected only few fully HPV16 pos cases, most likely not sufficiently powered to detect a survival advantage. However, the 7 E1+L1+E6+ non-OPC patients showed both better OS (77 months median) and PFS (median not reached) compared to E1-and/orL1+E6+ and HPV16-neg ones (32 vs 36 months for OS; 24 vs 36 months for PFS, respectively; P=NS). When each fragment was assessed individually neither E1 nor L1 nor E6 reached any significant difference in OS and PFS (**Figure 6**).

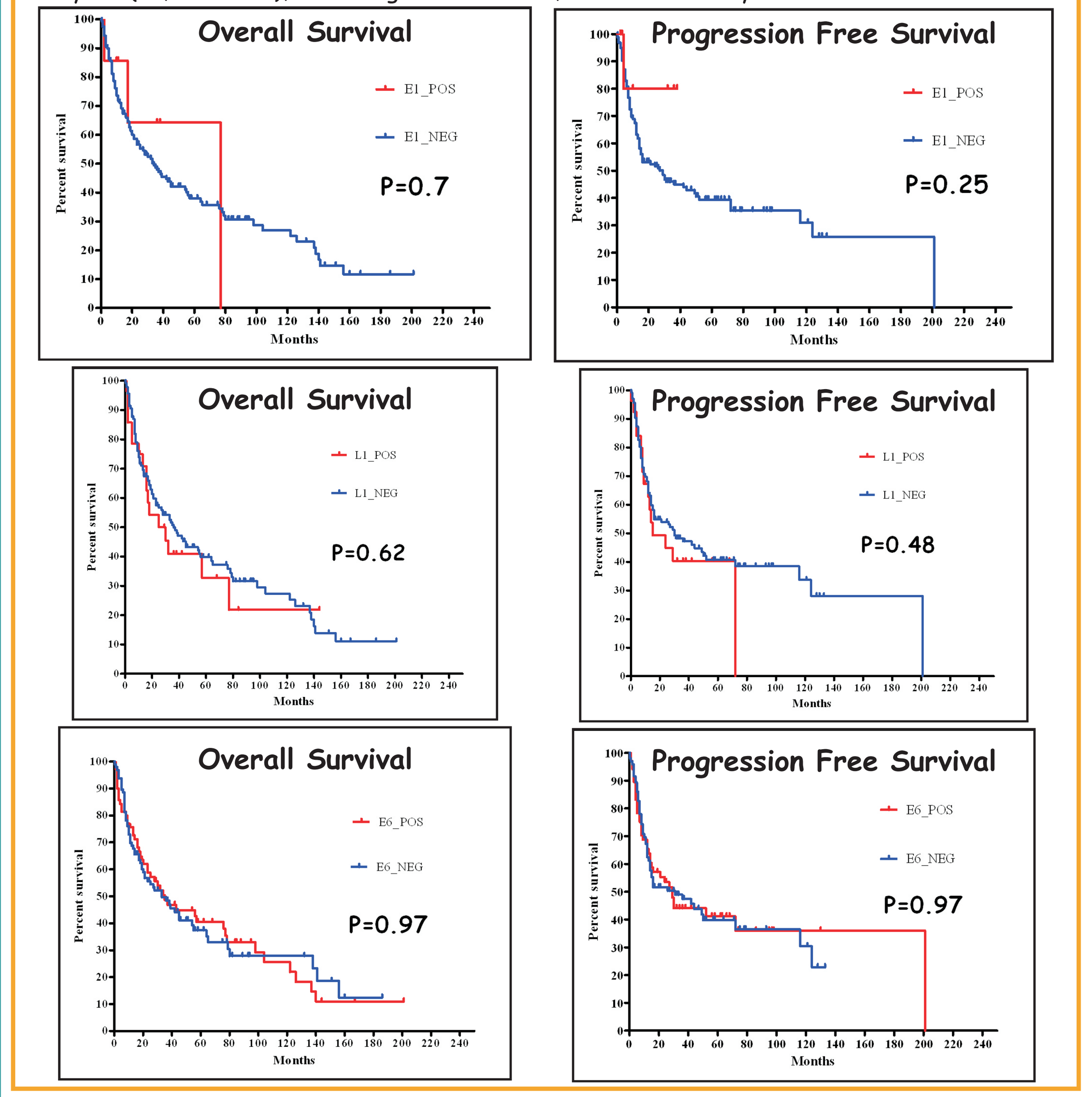

Figure 6. Kaplan-Meier curves in non-OPC patients (N=166) according with each viral fragment analyzed (E1, L1 and E6), according to viral status, taken individually for OS and PFS.

DISCUSSION

This study showed that HPV16 prevalence varied by use of 3 specific primer pairs targeting relevant viral sequences involved in oncogenesis (E6), capsidation (L1), viral replication and episomal maintenance (E1).

In our hands E6 showed the highest % of pos HPV samples compared to L1 and E1.

The viral fragment E6 plays a key role in malignant transformation and may be present in both HPV16 episomal and integrated forms, or as a persistent fragment that remains just a harmless DNA after a resolved infection. Moreover, as integration usually leads to disruption and deletion of E1 and E2 open reading frame (ORF), we may postulate that E1 detection tags the early and transcriptionally active viral infections.

In our study, the overall prevalence of HPV16 DNA positivity was higher in OPC patients (72%) compared to non-OPCs (43.4%), with the strongest association with E6 detection.

Instead, the lower viral prevalence found for L1 and E1 fragments might be due by their loss during the integration of viral genomic sequence into the host DNA.

Interestingly, in our study, E1 detection by DNA-PCR was always linked to E6 and L1 positivity and performed better in predicting the prognosis in OPC patients. Indeed, survival analysis according to DNA-PCR, showed that the pos E1 OPCs had both better OS (P=0.02) and PFS (P=0.053) than the neg ones. No other tumour condition, associated with viral fragment or/and status (E1 in non-OPC, L1 and E6 in OPC and non-OPC), taken individually or in combination, showed a significant difference in survival.

Thus, L1 and E6 detection by DNA-PCR, as individual biomarker, seems not informative in predicting prognosis in this series of patients.

This analysis corroborated E1 in OPCs as a strong prognostic marker for both OS and PFS than E6 and L1.

In conclusion, E1 by DNA-PCR in FFPE archival tissues, taken alone, is of clear importance in predicting survival and might represent a clinically valuable marker for the identification of OPC patients who have a better prognosis and may be candidate for deintensified treatments.

Future studies on non-OPC patients should be powered to address the clinical prognostic value of HPV status, eventually at each non-OPC primary sites.

REFERENCES

[1] Siegel R, Naishadham D, Jemal A, Cancer Statistics, 2012. CA Cancer J Clin 2012; 62: 10-29. DOI: 10.3322/caac.20138.

[2] Syrjänen K, Syrjänen S, Lamberg M, Pyrhönen S, Nuutinen J. Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. Int J Oral Surg 1983; 12:418-424. doi:10.1016/S0300-9785(83)80033-7.

[3] Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S, Viscidi R. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst 2008; 100: 407-420. doi: 10.1093/jnci/djn025.

[4] Isayeva T, Li Y, Maswahu D, Brandwein-Gensler M. Human papillomavirus in non-oropharyngeal head and neck cancers: a systematic literature review. Head Neck Pathol 2012; 6 Suppl 1: S104-S120. doi: 10.1007/s12105-012-0368-1. Epub 2012 Jul 3. Review.

[5] Tommasino M, Crawford L. Human papillomavirus E6 and E7: proteins which deregulate the cell cycle. Bioessays 1995; 17: 509-518. doi: 10.1002/bies.950170607

[6] Chiang CM, Ustav M, Stenlund A, Ho TF, Broker TR, Chow LT. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. Proc Natl Acad Sci U S A 1992; 89: 5799-5803.

[7] Venuti A, Paolini F. HPV detection methods in Head and Neck cancer. Head and Neck Pathol 2012; 6: S63-S75. doi: 10.1007/s12105-012-0372-5.

[8] Jordan RC, Lingen MW, Perez-Ordonez B, He X, Pickard R, Koluder M, Jiang B, Wakely P, Xiao W, Gillison ML. Validation of methods for oropharyngeal cancer HPV status determination in US cooperative group trials. Am J Surg Pathol 2012; 36: 945-954.. doi: 10.1097/PAS.0b013e318253a2d1.