

TLR4 down-regulation identifies high risk HPV integration in head and neck squamous cell carcinomas

Giuseppe Pannone¹, Pantaleo Bufo¹, Mirella Pace¹, Silvia Lepore², Giuseppe M. Russo¹, Corrado Rubini³, Renato Franco⁴, Gabriella Aquino⁴, Angela Santoro⁵, Giuseppina Campisi⁶, Vito Rodolico⁷, Eduardo Bucci⁸, Gennaro Ilardi⁹, Massimo Mascolo⁹, Francesco Merolla⁹, Lorenzo Lo Muzio¹⁰, Iole Natalicchio¹¹, Giuseppe Colella¹², Stefania Trino², Ilaria Laurenzana², Rosalia Leonardi¹³, Paolo Bucci⁸

¹Department of Clinical and Experimental Medicine, Section of Pathological Anatomy, University of Foggia, Foggia, Italy, ²Laboratory of Preclinical and Translational Research, IRCCS-CROB, Oncological Reference Centre of Basilicata, Rionero in Vulture (PZ), Italy, ³Section of Pathological Anatomy, Polytechnic University of Marche, Ancona, Italy, ⁴Section of Pathological Anatomy, National Cancer Institute IRCCS 'G. Pascale' Foundation, Napoli, Italy, ⁵Department of Laboratory and Services, Section of Histopathology, Research and Care Foundation 'Giovanni Paolo II', Catholic University of Sacred Heart (UCSC), Campobasso, Italy, ⁶Department of Surgical, Oncological and Stomatological Disciplines (Di.Chir.On.S.), University of Palermo, Via del Vespro 129, 90127 Palermo, Italy, ⁷Department of Sciences for the Promotion of the Maternal and Childhood Health "G. D'Alessandro", Section of Anatomic Pathology, University of Palermo, A.O.U. Policlinico "P. Giaccone", Palermo, Italy, ⁸Department of Neurosciences and Reproductive and Odontostomatological Sciences, University of Napoli 'Federico II', Napoli, Italy, ⁹Department of Advanced Biochemical Sciences, Section of Anatomic Pathology, University of Napoli Federico II, Via S Pansini 5, Napoli, Italy, ¹⁰Department of Experimental and Clinical Medicine, Dental Clinic, University of Foggia, Foggia, Italy, ¹¹Section of Clinic Pathology, OO.RR., Foggia, Italy, ¹²Department of Head and Neck Surgery, Second University of Naples, Naples, Italy, ¹³Department Medical-Surgical Specialties, Section of Oral Medicine, Policlinico, University of Catania, Catania, Italy

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1. ABSTRACT

TLRs are main actors of the innate immune response against HPV. There are very few studies on

the role of TLRs mediated HPV clearance in Head and Neck oncology. Our aim was to evaluate whether TLR4

expression identifies HPV infection and/or HR-HPV integration status in oral and oropharyngeal cancers. By immunohistochemistry we assessed TLR4 levels in OSCC. To detect viral integration or episomic status *In situ* hybridization for HPV-DNA and Pyro-sequencing techniques have been performed. The relationship between TLR4 expression with HPV infection status has been investigated. ISH HPV positive samples have reported lower levels of TLR4 intensity than negative samples ($p = 0.002$). There is no statistical correlation between TLR4 intensity and PCR HPV results ($p > 0.05$). Point-biserial correlation coefficient revealed significant association between TLR4 expression and HR-HPV integration status ($p = 0.0001$) and between TLR4 expression index and HR-HPV infection ($p = 0.001$). These data show that TLR4 down-regulation is strongly associated to both HPV-16 infection and its integration into the host DNA.

2. INTRODUCTION

On a global scale head neck cancers account for about 10 percent of all malignant tumors in men and 4 percent in women. Ninety percent of head neck cancers are represented by oral squamous cell carcinoma (OSCC) that to world level, represents the fifth tumor for incidence and the sixth cause of mortality for cancer (1). Cigarette smoking, alcohol, chewing of betel nuts, low consumption of fruit and vegetables, reduced immunity and the infection of high-risk Human Papilloma Viruses (HR-HPVs) are the main risk factors for the development of OSCCs. During the past 20 years, the incidence of HPV-associated oropharyngeal cancer is increased. It has been estimated that HPV will cause more oropharyngeal cancers than cervical cancers in the United States within 2020 (2). HPVs infect human skin and mucous membrane, through direct epithelial-epithelial contact. More than 120 different HPV genotypes have been identified and almost 45 subtypes have been classified in Low-risk HPVs (LR-HPVs), and High-Risk (HR-HPVs) types, considering their potential risk to induce an invasive cancer. In particular HPV types 16 and 18 are responsible for the majority of HPV-caused cancers.

The natural history of HPV infection is strongly influenced by the equilibrium between host and infecting agents. In most cases the viruses are eliminated by immune system, in other cases they can remain latent or undergo to self-replication. HPV, especially the HR group, has developed a series of mechanisms to escape the host's defense system, particularly eluding the innate immunity and inducing a delay in the activation of the adaptive immunity (3). Principal actors of the innate immune response are represented by the TLRs (Toll like receptors) and different studies have reported that HPV can directly inhibit the functions of the TLRs pathway through interferons (IFNs) (4). TLRs are trans-membrane proteins, present on a variety of cells types, able to

recognize invading microbes and to activate signaling pathways that in turn launch immune and inflammatory responses to destroy the invaders (5,6). The TLR family includes thirteen proteins (TLR1–TLR13), consisting of an extracellular domain with leucine-rich repeats, a trans-membrane region and a cytoplasmic tail, called TIR (Toll-IL-1R (Interleukin-1-Receptor)). The core pathway utilized by most TLRs leads to activation of the transcription factor Nuclear Factor- κ B (NF- κ B), which in turn regulates the production of inflammatory cytokines and chemokines. In this way, TLRs play important roles in immune processes: firstly, they drive cytokine and chemokine production (TNF, INF, IL-1, IL-2, IL-6, IL-8 IL-12); then, they have an important role in adaptive immunity by activating antigen presenting cells (APC) (5).

The interaction between HPVs and TLRs has been underlined by both *in vitro* and in animal models (7,8) but there are very few studies on the role of TLR mediated HPV clearance in human pathology (9), being the most part of the data produced in the gynecological field. Anyway, studies on TLR4 expression in tumor cell lines demonstrate that TLR-4 in cell lines derived from melanoma (B16), colon cancer (MC26), breast cancer (4T1), lung cancer (LLC1) and prostate cancer (RM1) (10). As regard TLR4 and epithelial HPV-unrelated cancer there are numerous studies in current literature regarding prostate cancers (11,12), breast cancers (13,14), ovarian cancers (15,16), gastric cancers (17), colorectal cancers (18), lung cancers (19,20) and melanoma (21).

An indirect relationship between the role of TLRs and HPV-related uterine cervical cancer has been reported by L. Yu *et al.* (22). Results from this study showed a decrease in the expression of TLR4 during the progression of cervical neoplasia and down-regulation of TLR4 appeared to be associated with the expression of P16^{INK4A}, which in turn is a crucial marker of HPV integration into host cells.

Regarding oral mucosa, several studies have described the expression patterns of various TLR both in inflamed tissues that in healthy periodontal tissues (23-29). These studies suggest that TLR4 has a strong expression in inflamed tissues (26,28,29) and appears to be constitutively expressed in gingival epithelial cells in culture (26,30-32) and in epithelial and connective tissue of the healthy gums and periodontitis.

Furthermore, a recent study (33) has evaluated TLR4 signaling effects on human head and neck squamous cell carcinoma (HN-SCC). Authors have shown the association between TLR4 expression intensity and histological tumor grade. Moreover, they affirmed that the lipopolysaccharide (LPS) binding to TLR4 on tumor cells enhanced proliferation, activated phosphatidylinositol 3-kinase/Akt pathway, up-regulated IRAK-4 expression,

Table 1. Clinicopathological data of the studied population

	Numbers	Percentage
Sex		
M	67	72.8
F	25	27.2
Age		
Mean	64.4	
Range	25-100	
Sites		
Tongue	55	59.8
Tonsil	13	14.1
Gum	3	3.3
Cheek	2	2.1
Oral cavity n.s.	2	2.1
Tongue and floor	2	2.1
Rhino-Oropharynx	4	4.3
Commissura	1	1.1
Upper lip	1	1.1
Floor of mouth	1	1.1
Trigonus	1	1.1
Palate	1	1.1
Base of tongue and pillar	1	1.1
Trigonus and pillar	1	1.1
Trigonus pillar and palate	1	1.1
Hypopharynx	3	3.3
Original variants diagnosis		
Keratinizing OSCC	86	93.5
Basaloid OSCC	2	2.1
Papillary OSCC	1	1.1
Verrucous OSCC	1	1.1
Verrucous-squamous hybrid OSCC	1	1.1
Basaloid squamous OSCC	1	1.1
Grade		
G2	35	38.0
G3	30	32.6
G1	19	20.7
n.d.	8	8.7
Total cases: 92, Abbreviations: n: number of cases; n.s.: not specified; n.d.: not determinate.		

induced nuclear NF- κ B translocation and increased production of interleukin IL-6, IL-8, VEGF, and granulocyte

macrophage colony-stimulating factor. They also revealed that TLR4 triggering protected tumor cells from lysis mediated by NK-92 cells, concluding that TLR4 ligation on tumor cells supports HN-SCC progression (33).

Furthermore, it has also been demonstrated that the development of cisplatin drug-resistance in human OSCC might occur through the mechanism involving TLR4 and its signaling pathway (34). Since up to now, only few studies were conducted on the interactions between TLR and HPV in HN-SCC (33,34), our study aim has been to determine the relationship between TLR4 expression and HPV infection and in a second time to evaluate whether TLR4 identifies HR-HPV integration state in OSCC.

3. MATERIALS AND METHODS

3.1. Study cases

In this retrospective study, paraffin blocks from HN-SCC resections were retrieved from the archives of Section of Anatomic Pathology - University of Foggia (Foggia, Italy), Section of Anatomic Pathology - National Cancer Institute IRCCS 'G. Pascale' Foundation of Naples (Naples, Italy), Section of Anatomic Pathology - University of Palermo (Palermo, Italy), Section of Anatomic Pathology - University 'Federico II' of Naples (Naples, Italy), Section of Anatomic Pathology - Polytechnic University of Marche, Ancona (Ancona, Italy). A single block for each case was selected for use. Tissues from ninety-two patients were collected in a retrospective period of 21 years (1992-2013). The study was approved by the Research Ethics Boards of Fondazione 'G. Pascale', Naples, Italy. All the patients included in the study have been treated according to standard diagnostic and therapeutic criteria currently approved at the time of therapy. Briefly, for oral cancers trans-oral resections plus sentinel lymph-node ablation have been performed for T1-2/N0 while resection of primary plus neck lymph-node dissection (levels I-IV; for any T with N+, resection of T plus neck lymph-node dissection) have been made for T3-4/N0. For maxillary and ethmoidal tumors of any T, total maxillectomy or extended maxillectomy was performed while for the same tumours of any T with N+ total maxillectomy or extended maxillectomy plus neck lymph-node dissection (levels I-IV) were made. Adopted criteria for adjuvant therapy after primitive cancer removal and neck dissection were the following: i) positive margins or close (<5 mm); ii) T3-T4 primary tumor; iii) vascular invasion; iv) peri-neural invasion; v) N>1 (more than one positive lymph-node including micro-metastasis and extracapsular spread). Clinicopathological data of the study population were resumed in Table 1.

3.2. Immunohistochemistry on Tissue Micro-Array (TMA-IHC)

H&E staining of a 4- μ m TMA section was used to verify all samples. Sections (4 μ m) were cut

from formalin-fixed, paraffin-embedded blocks and they were mounted on glass slides coated with poly-L-lysine, dried at 37°C overnight, de-paraffinized in xylene, washed in ethanol, and finally washed in PBS (pH 7.4.). Immunohistochemical (IHC) analysis on 4-µm TMA serial sections was performed by using Ventana Benchmark® XT autostainer and standard linked streptavidin-biotin horseradish peroxidase technique (LSAB-HRP), according to the best protocol for the antibody used in our laboratory.

Primary mouse monoclonal antibody anti-TLR4 was diluted 1:300 in PBS (code NB 100-56566 clone 76B357.1. Novus Biologicals) and incubated overnight. Negative control slides without primary antibody were included. Immunoreactions products were assessed following incubation at room temperature for 5–10 min with *iVIEW DAB Detection Kit* (Ventana Medical Systems). Slides were counterstained with type II Gill's Haematoxylin, and then examined by light microscopy.

The results of the IHC staining were evaluated separately by two observers (GP, AS). In each tissue section 10 representative high power fields (HPFs) were analysed at optical microscope (Nikon Eclipse 1000, at x40) and were selected for TLRs positive tumor cells with an average of 2,000 tumour cells per case. The topographical staining pattern was also evaluated and recorded as membranous (M), cytoplasmic (C), or mixed. For each case, the cumulative percentage of positive cells among all sections examined was determined. Since till now there is not standardized criteria for TLR staining evaluation, we have chosen to grade and score the extent of TLR4 immunostaining as follows: (0) points for negative staining of the considered cells, (score 1) <10%, (score 2) 10-50%, (score 3) 51-80% and (score 4) ≥ 80% positive staining of the evaluated cells. Staining intensity has been scored as follow: negative, faint, 1+, 2+, 3+. Inter-rate reliability between the two investigators blindly and independently examining the immunostained sections was assessed by the Cohen's K test, yielding K values higher than 0.7.0 in almost all instances.

3.3. *In situ* hybridization (ISH) for HPV-DNA detection in morphological context

A commercially available HPV ISH system has been used (Ventana Inform HPV, Tucson, AZ, USA) to detect integration or episomic status in our study (35). Briefly, ISH was performed using the *Benchmark*® plate and an alkaline-phosphatase coupled antibody detection method. The hybridization signals were shown with Tetrazole Blu and Fast Red nuclear counterstaining. The commercially available Ventana kits include the following probes for HR-HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66 (Inform HPV family-III 16 Probe; Ventana - Roche); and the following probes for LR-HPVs, 6, 11 (Inform HPV family- II 6 Probe; Ventana – Roche).

3.4. HPV-DNA detection. Pyro-sequencing method

For the formalin-fixed, paraffin-embedded (FFPE) samples, 10 x 10 µm tissue sections were deparaffinized in xylene at 55°C for 5 h, dehydrated in ethanol and dried overnight, followed by incubation in digestion buffer (proteinase K, 10 mM Tris.Cl (pH 8.0.), 1 mM EDTA (pH 8.0.), 1% SDS) at 55°C for 3 h and overnight at room temperature. The DNA was phenol extracted and precipitated with 100% alcohol in the presence of 0.3. M sodium acetate (pH 5.2.). GP5+/6+ consensus primers (36) that are known to recognize more than 30 different HPV types were used to amplify a region of the L1 major capsidic protein of HPV from the samples. The reverse primer GP6+ used in the reactions was biotinylated. The PCR reaction mixture (1X Taq PCR buffer (Invitrogen), 2.5. mM MgCl₂ (Invitrogen), 200 nM of each dNTP, 1 µM of each primer and 5 U of Taq polymerase) was subjected to denaturation at 94°C for 1 min, 40 cycles at 94°C, 1 min; 40°C, 2 min and 72°C, 1.5. min, followed by final extension at 72°C, 5 min. The biotinylated PCR products were purified from the reaction mixture by immobilizing onto streptavidin-coated sepharose beads on the vacuum-based workstation provided with the BIOTAGE PSQ 96MA instrument in a 96-well plate format as described (37). Briefly, the DNA was denatured in 100 mM NaOH, mixed with 15 pmol of the seven-HPV primers mix and incubated at 80°C for 3 min followed by slow cooling to room temperature, in the presence of annealing buffer (10 mmol/l Tris-acetate (pH 7.7.5), 5 mmol/l Mg acetate). The primer pool used for the detection of high-risk HPV types 16, 18, 31, 33, 35 and low-risk types 6 and 11 contained the oligonucleotides as previously described (36). The sequencing reactions were set up in 96-wellplates with 6 cycles of sequential dispensation of dATPαS, dCTP, dGTP and dTTP.

3.5. Statistical analysis

Data have been analyzed utilizing MedCalc 12.2.1.0. (for Windows), SOFA Statistics 1.4.3. and R 2.1.1.1. (for Linux) statistical softwares using Debian 7 and Windows Operating Systems. Statistical evaluations were carried out using Fisher Exact Probability Test to assess differences between ISH and PCR HPV test and Mann Whitney U test to compare the TLR-4 intensity with ISH and PCR HPV results. Correlation between immunohistochemical staining intensity for TLR-4 (from zero to 3+) and HPV integration (1 = HR-HPV integration positive signals; 0 = negative ISH for HR-HPV) has been investigated by Point-biserial correlation coefficient. In order to select a relevant IHC cut-off score to identify HPV positive samples, Receiver Operating Characteristic (ROC) curve analysis was carried out. At each IHC score, the sensitivity and specificity of TLR4 expression for the ISH results was plotted, generating a ROC curve. Criterion values and coordinates of the ROC curves for TLR4 expression were described in Table 2. The point on the curve, maximizing sensitivity and specificity of TLR4

Table 2. TLR4 expression in OSCC. Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	- LR	+PV	-PV
<0	0.00	0.0-30.8	100.00	93.4-100.0		1.00		84.4
≤0	80.00	44.4-97.5	92.59	82.1-97.9	10.80	0.22	66.7	96.2
≤2	80.00	44.4-97.5	29.63	18.0-43.6	1.14	0.68	17.4	88.9
≤2.5	90.00	55.5-99.7	27.78	16.5-41.6	1.25	0.36	18.8	93.7
≤3	100.00	69.2-100.0	0.00	0.0-6.6	1.00		15.6	

CI: confidence interval; +LR: positive likelihood ratio; -LR: negative likelihood ratio; +PV: positive predictive value; -PV: negative predictive value

Table 3. TLR4 expression index in OSCC. Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	- LR	+PV	-PV
<0	0.00	0.0-30.8	100.00	93.4-100.0		1.00		84.4
≤0	80.00	44.4-97.5	92.59	82.1-97.9	10.80	0.22	66.7	96.2
≤170	80.00	44.4-97.5	38.89	25.9-53.1	1.31	0.51	19.5	91.3
≤180	90.00	55.5-99.7	37.04	24.3-51.3	1.43	0.27	20.9	95.2
≤200	90.00	55.5-99.7	25.93	15.0-39.7	1.21	0.39	18.4	93.3
≤225	100.00	69.2-100.0	25.93	15.0-39.7	1.35	0.00	20.0	100.0
≤300	100.00	69.2-100.0	0.00	0.0-6.6	1.00		15.6	

CI: confidence interval; +LR: positive likelihood ratio; -LR: negative likelihood ratio; +PV: positive predictive value; -PV: negative predictive value

intensity was selected as the cut-off score above which TLR4 expression was considered positive marker of HPV positivity. The expected average value of the ROC area is 0.5, if there is no discrimination between the groups. In order to distinguish a real discrimination between the groups from the case of no discrimination, a p-value was calculated. A small p-value makes it unlikely that the ROC area can be reconciled with the case of no discrimination. Only values of $p < 0.01$ were considered significant. The same ROC curve analysis was performed on TLR4 expression index related to HPV positivity (Table 3).

4. RESULTS

Table 1 resumes the clinicopathological data of the study population. ISH was performed in 86 cases: 76 stained negative (88,4%) and 10 (11,6%) positive, showing a clear integrated pattern or both clusters and integration signals in 45,5% and focal clusters in 10% of the examined cases. PCR detected HPV-16 type in 11 carcinomas (but only 6 cases stained positive at ISH). One case harbored HPV-31 (and showed negative ISH), one case resulted positive for HPV53 and one had a double HPV infection for HPV-31 and HPV-44 (ISH showed cytoplasmic positivity for the probe, but never nuclear). Fisher Exact Probability Test was performed to evaluate the differences between the results on the same sample by ISH and PCR. There are statistically significant differences between ISH and PCR ($p < .001$; Phi = +0.6.) (Figure 1).

IHC evaluation of TLR4 staining was performed by two pathologists (GP and AS). Seven (7.6%) SCCs stained as negative, 9 (9.8%) as faint, 10 (10.9%) as 1+, 30 (32.6%) as 2+ and 21 (22.8%) as 3+; 15 (16.3%) cases showed heterogeneous staining intensity ranging from negative to 3+. Overall cancer cells showed cytoplasmic expression with only sporadic membrane staining.

TLR4 always stained both dysplastic and apparently normal epithelium surrounding cancer in the 79 sections where evaluable normal epithelium was detected by observers. Intensity of immunostaining varied from 1+ to 3+ and involved almost the basal lines, but could also affect the whole thickness of the epithelium.

In ISH HPV positive cases for which it was possible to evaluate normal epithelium surrounding tumors (6 cases), the limit of transition between the normal mucosa and carcinoma was clearly delineated by the negativity of TLR4 in 50% of the cases or by reduction of the staining towards the faint intensity.

ISH HPV positive samples reported lower TLR4 intensity (median: 0; lower quartile: 0; upper quartile: 2.0.) than negative samples (median: 2.0.; lower quartile: 1.0.; upper quartile: 2.0.) (Figures 2 and 3). Mann Whitney U test confirmed this statistically significant difference ($p = .002$) with a median of TLR4 intensity of 2.0. for ISH negative samples and a median of TLR4 intensity of 0

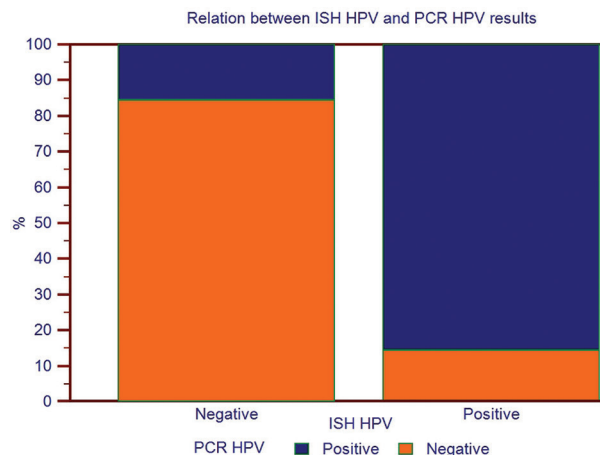


Figure 1. Relation between ISH HPV and PCR HPV results. This figure shows the differences between the results on the same sample by ISH and PCR. ($p < .001$; $\Phi = +0.6$)

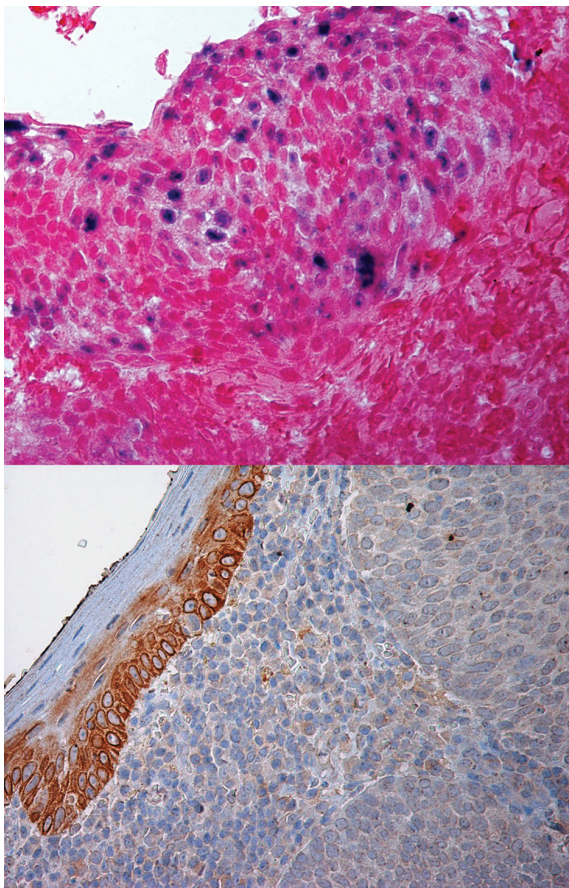


Figure 2. HR-HPV-positive OSCCs show strong down-regulation of TLR4. Note strong down-regulation of TLR4 in OSCC when compared to surrounding epithelium. ISH for HR-HPV shows integrated HPV in the tumor and lack of expression in surrounding epithelium expressing TLR4. (IHC staining, LSAB-HRP, nuclear counterstaining with Gill's type-II haematoxylin; HR-HPV ISH, INFORM HPV III Family 16 Probe; Ventana – Roche; LR-HPV ISH, Inform HPV Family II 6 Probe; Ventana – Roche).

for ISH positive group. Instead, there was no statistical correlation between TLR4 intensity and PCR HPV results ($p > 0.05$).

Then, TLR4 intensity has been multiplied for IHC staining percentage in order to obtain TLR4 expression score ranging from zero to 300 units. Mann Whitney U test also confirmed that ISH HPV positive group presented lower TLR4 expression index ($p = .002$) with a median of 140 for ISH negative samples and a median of 0 for ISH positive group ($p = .001$). No statistical correlation was found between TLR4 expression score and PCR HPV results ($p > .05$).

IHC staining intensity for TLR4 (from zero to 3+) has been compared to HPV integration (1 = HR-HPV integration positive signals; 0 = negative ISH for HR-HPV).

This correlation has been investigated by Point-biserial correlation coefficient. Statistically significant relationship between TLR4 expression and HR-HPV integration has been found ($p = .0001$; $r = -0.4.1$; 95% CI for r : -0,572 to -0,217) and between TLR4 expression score and HR-HPV infection ($p = .001$; $r = -0.3.5$; 95% CI for r : -0,522 to -0,148) (Figure 4).

In addition, ROC curve analysis was performed to identify a possible cut-off of TLR4 intensity for HPV positivity. The most significant cut-off was TLR4 staining intensity equal to zero for ISH HPV positivity. The area under the ROC curve was 0.813 (95% CI: 0.6.96 to 0.9.00; Youden index J: 0.7.26; $p = 0.0.022$;) and associated cut-off criterion was set to TLR4 intensity equal to zero (Sensitivity, 80.0.%; Specificity 92.6.%; Positive Predictive Value, 66.7.%; Negative Predictive Value, 96.2.%).

The same ROC curve analysis was performed on TLR4 expression index related to HPV positivity (Table 3). The most significant cut-off was TLR4 expression index equal to zero for ISH HPV positivity. The ROC area was 0.8.34 (95%CI: 0.7.20 to 0.9.15; Youden index J: 0.7.26; $p = 0.0.002$;) and associated cut-off criterion was set to TLR4 intensity equal to zero (Sensitivity, 80.0.%; Specificity 92.6.%; Positive Predictive Value, 66.7.%; Negative Predictive Value, 96.2.%) (Figure 5).

5. DISCUSSION

5.1. TLR expression in OSCC and clinic-pathological data

TLR4 expression has been previously reported in few papers regarding HN-SCC. Interestingly, by real-time PCR or PCR-RFLP method, Zeljic K *et al.* studied association among TLR2-3-4 and CD14 genes polymorphisms with oral cancer risk and survival (38). Authors stated that a significant increase in oral cancer risk was observed in individuals with mutated genotype of

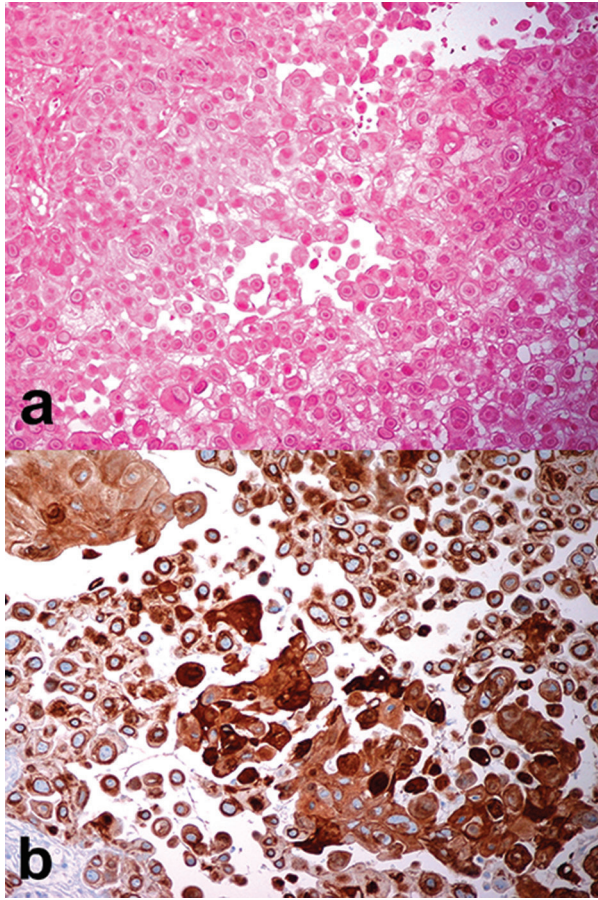


Figure 3. HR-HPV-negative OSCCs are TLR4 positive. A case of aggressive acantholytic OSCC showing negative staining for HR-HPV ISH (A), and strong immune reaction for TLR4 (B); (IHC staining, LSAB-HRP, nuclear counterstaining with Gill's type-II haematoxylin; HR-HPV ISH, INFORM HPV III Family 16 Probe; Ventana – Roche; LR-HPV ISH, Inform HPV Family II 6 Probe; Ventana – Roche).

TLR3 rs3775291 polymorphism (OR = 1.0.96, P = 0.0.36) compared to wild-type. They also demonstrated that the heterozygous and mutated genotype of TLR3 rs5743312 polymorphism could be considered as potential predictor of worse overall survival in advanced (stage III) oral cancer, but not independently of nodal status. Moreover, in the same study Authors reported TLR4 expression in 74% of HN-SCCs but they did not found correlation to clinic-pathological parameters and to patient survival; the association to the HPV-status has been not investigated. In contrast, Sun *et al.* (34) and Szczepanski *et al.* (33) reported a statistically significant association with histopathological degree in OSCC and HN-SCC, respectively. In our work, we have shown that TLR-4 down-regulation is not related to the histological degree of differentiation but rather related to HPV-16 infection and to HPV integration into the host DNA. Therefore, our study shows that OSCCs can be divided into two groups, considering the expression levels of TLR4: one that match with non-keratinizing histotype and HPV positivity,

and the second one matching with keratinizing histotype and HPV negativity, corresponding with negative/low and moderate/high TLR4 expression, respectively.

5.2. Microscopic diagnosis and HPV

As regards microscopic diagnosis, our study population included the following histotypes:

- 2 (2.1.%) non keratinizing (basaloid) carcinomas (one involving trigonus and anterior pillar and one from base of tongue);
- 88 (93, 6%) classical keratinizing HN-SCCs;
- 4 (4.2.%) keratinizing variants: one (1.0.6%) *basaloid-squamous* hybrid, involving anterior pillar and base of the tongue, one (1.0.6%) *papillary* SCC of the tongue, one (1.0.6%) *verrucous* carcinoma from tongue and one (1.0.6%) *verrucous-squamous* hybrid carcinoma of the tongue.

All the HN-SCCs were revalued considering recent acquisitions in terms of morphology (39). Twelve carcinomas differed from previous diagnosis of keratinizing squamous cell carcinomas (SCCs). Regarding HPV status, 5 cancers were positive for HR-HPV genotypes infection by ISH or PCR (see Table 2).

Considering overall, both the variants identified with recent microscopic observations and those ones previously diagnosed, we have:

- 5 basaloid not keratinizing carcinomas (5,3%);
- 89 squamous keratinizing HN-SCCs (94,68%).

Among the squamous keratinizing cancers we have recognized the following variants:

- 76 cases of classical keratinizing SCCs;
- 7 cases of basaloid-squamous hybrid carcinomas;
- 3 cases of verrucous-squamous hybrid carcinomas;
- 2 cases of verrucous carcinoma;
- 1 case of papillary carcinoma, representing respectively 80.8%, 7.4%, 3.2%, 2.1% and 1.0.6% of the entire study population and 85.3.9%, 7.8%, 3.3.7%, 2.2.4% and 1.1% of the keratinizing SCCs.

5.2.1. Basaloid variant

The group of basaloid carcinomas consisted of 2 OSCCs (from floor of mouth, and trigonus), 1 OP/OSCC (from trigonus and anterior pillar) and 2 OPSCCs (one from tonsil and one from base of tongue). Two cases were HR-HPV ISH positive but PCR negative. One case was PCR positive for HPV-16 infection but HR-HPV ISH negative. Overall 3 cases (60%) of basaloid HNSCCs were positive for HR-HPV infection, with a lower frequency than reported by other Authors (40). Westra *et al.* suggested to consider oropharyngeal basaloid HR-HPV HNSCC as a well differentiated variant of squamous carcinoma resulting from neoplastic transformation of the tonsillar crypts epithelium (41,42). The 'basaloid' aspect of HPV-related HN-SCC may induce confusion with poorly differentiated cancers (42). Considering

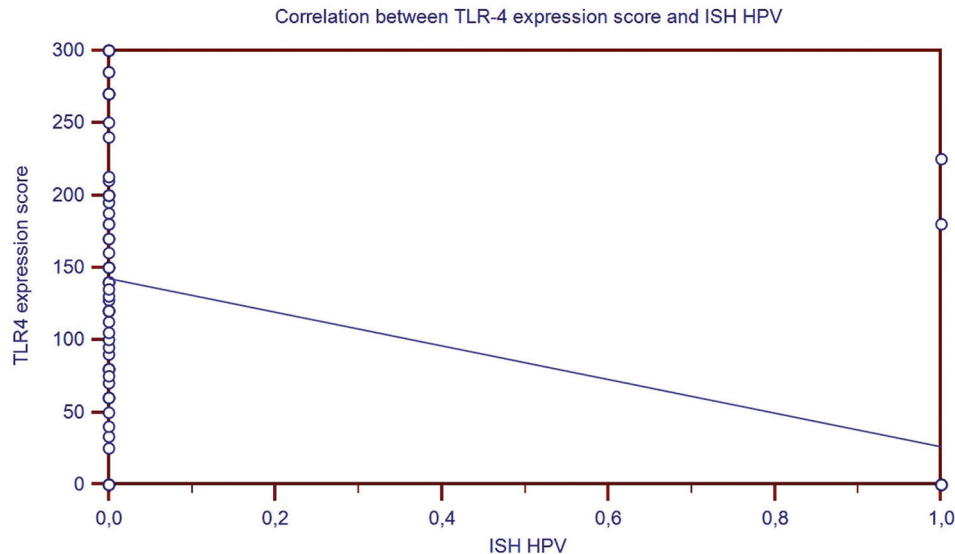


Figure 4. Correlation between TLR-4 expression score and ISH HPV. TLR4 expression score has been compared to HPV integration (1 = HR-HPV integration positive signals; 0 = negative ISH for HR-HPV). Statistically significant relationship between TLR4 expression score and HR-HPV infection.

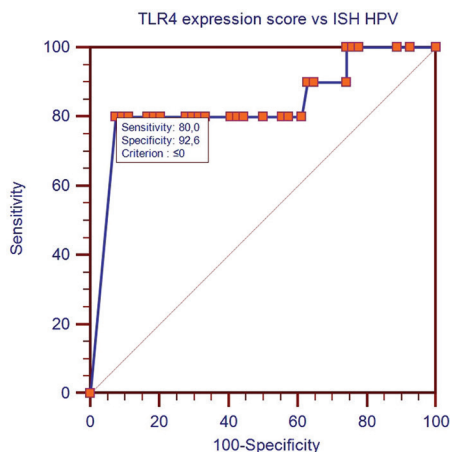


Figure 5. ROC curve on TLR4 expression score vs ISH HPV. ROC curve analysis was performed to identify a possible cut-off of TLR4 expression score related to HPV positivity. The most significant cut-off was TLR4 expression score equal to zero for ISH HPV positivity. The ROC area was 0.8.34 (95%CI: 0.7.20 to 0.9.15; Youden index J: 0.7.26; $p = 0.0.002$).

this, we can conclude that in our case series there were 2 OPSCCs with basaloid morphology, only one of them showing integrated nuclear pattern with HR-HPV ISH while the second cancer was not related to HPV.

5.2.2. Classical keratinizing SCCs

Among 76 keratinizing SCCs, twelve (15.7%) cases were HR-HPV related considering both ISH determination and PCR evaluation. The last method revealed HPV-16 infection in nine (11.8%) of them, HPV-31 in two (2.6%) of them and HPV-53 in one (1.3%) of the keratinizing carcinomas.

5.2.3. Basaloid-squamous variant

Of the seven basaloid-squamous carcinomas, 5 were OSCCs (involving multiple sites of oral cavity in one case, three cases of tongue cancer and one extending from tongue to the floor of the mouth), 1 was OP/OSCC localized to base of tongue and pillar and 1 OPSCC of to the tonsil. Three cases harbored HPV-16 infection and one resulted positive also by HR-HPV ISH. Overall, we detected HR-HPV in 3 cases (42.8%) of basaloid-squamous variant. Also this morphological variant was associated with HPV infection (40).

5.2.4. Verrucous-squamous variant

The verrucous-squamous hybrid group consisted in 3 cases of OSCCs, two localized to the tongue and one to multiple sites of oral cavity. All three cases were HR-HPV ISH negative but two of them harbored HPV 16 as reported by PCR based typing.

5.2.5. Verrucous variant

As regards two verrucous carcinomas of the tongue, we had information only about HR-HPV ISH status, and not about HPV infection status by PCR.

5.2.6. Papillary variant

ISH detected the presence of HR-HPV in the papillary OSCC of the tongue and PCR revealed HPV-16 positivity. The complete down-regulation of TLR-4 in OP-OSCC occurs in the presence of integrated HPV-16 and it is not a prerogative of all HPV viruses as the LR-HPVs are not able to reduce the expression of TLR4; in addition also HR-HPV viruses non 16 are not able to significantly reduce the TLR-4. Among the non 16 HR-HPVs, HPV-31 was the second most frequent type

detected in OPSCC and it shows a moderate ability to reduce the intracellular levels of TLR4.

Interesting results of our study on the selective down-regulation of TLR4 in the HPV-16 related OSCC, when compared with LR-HPVs associated OSCCs and not type-16 HR-HPVs OSCCs, demonstrated that the ability to evade the immune system by the basal cell of reticulated epithelium of the tonsillar crypts, from which HPV-related OSCCs give rise, depends on the intrinsic potency of HPV-16. In line with this concept, previous studies on innate immunity performed on cell lines of cervical carcinoma containing different types of integrated HR-HPVs and relatively different viral loads have shown that TLR4 mRNA is strongly expressed in HPV negative uterine cervix cell lines (C-33A), while it is strongly down-regulated in cell lines with HPV-16 integrated at high viral load (Caski), and in line with HPV-68 (ME180), and moderately down-regulated in lines containing HPV-18 (HeLa) and in lines carrying HPV-16 in low copy number (SiHa) (43,44). This suggests that different HR-HPVs and their relative copy numbers affect the cell's ability to down-regulate TLR-4, in this way promoting the escape from the host immune system.

5.3. TLR-4 and chemo-resistance

It is well known that oral cancer unrelated to HPV is generally quite resistant to chemotherapeutic agents; on the other hand, HPV-related OSCC are characterized by radio-chemo sensitivity. It is not yet clear, however, the potential role of TLRs in the phenomena of chemo-radio-resistance. Recently two works on cell lines from oral-SCC (34) and HN-SCC (33) have shown that the stimulation with LPS, activating TLR4, increases the resistance to cisplatin-induced apoptosis. In this perspective, TLR4 suppression might increase cisplatin sensitivity, thus improving the prognosis of OSCC patients. In particular, Z. Sun *et al.* (34) have demonstrated that LPS significantly lowered cisplatin-induced apoptosis by elevating the resistance of OSCC cells but not HIOEC cells (human immortalized oral epithelial cells, obtained from normal oral mucosa immortalized by transfection of HPV16 E6/E7 gene). In our study, TLR4 is over-expressed in HPV-unrelated OSCCs while it is absent in HPV-16-related OSCCs. Therefore, our work provides an additional molecular proof in agreement with the well-documented radio-chemo sensitivity of HPV-related OSCCs and the higher levels of chemo-resistance of oral cancer unrelated to HPV.

5.4. TLR4 down-regulation and HPV immune surveillance evasion

In the gynecopathological context TLRs play a fundamental role in the clearance of HPV in uterine cervix infection. Furthermore, time to progression from pre-cancerous lesion to invasive cancer may induce immune tolerance (45,46). Several cancers tissue and cell lines showed a strong expression of TLR4 and this

is interpreted as protective for tumors because of its role in immune surveillance evasion, proliferation and survival (10,16,47–51). But, consistent with our results, carcinomas of the uterine cervix show a decrease in TLR4 expression if compared with normal epithelium (22). This could be related to the role of the virus as causative agent of cervical cancer. The persistence of HPV infection is the first step for HPV DNA integration and then for transformation of epithelial cells. HPVs are able to elude both innate and adaptive immune response and can avoid recognition by Antigen Presenting Cells (APC) (3,52).

In addition, HPV-related cancers are very common in immune-deficient patients, with a worse prognosis compared to HPV-related cancers developing in immune-competent individuals (53). It seems that early proteins have a predominant role in the evasion of the immune defense. It is clear that in HPV-16 and HPV-18 transformed cells (SiHa, CaSki and HeLa), MHC class I (Major Histocompatibility Complex) is down-regulated, in fact when transfected with E7-specific siRNA, inhibiting transcription of the early gene, the cells show a statistically significant growth of MHC class I, while the tetracycline induction of HPV16- E7 reduces their expression (54). Likewise, several Authors proved that E6 and E7 are involved in TLR4 down-regulation. In fact, in vaginal, ecto-cervical and endo-cervical cell lines, immortalized with HPV-16 E6/7 (VK2/E6E7; Ect1/E6E7; End1/E6E7) and assayed for TLRs mRNA expression and by flow cytometry for several TLRs, only TLR4 showed a low surface expression and a decrease in levels of primary transcripts (55). Furthermore, HIOEC oral cells immortalized with HPV-16 E6/E7 transfection have very low expression level of TLR4 and MyD88 (34). Preliminary results in equine fibroblasts culture infected with Bovine Papillomavirus type1, demonstrate a decrease in TLR4 mRNA levels when E7 and E2 were expressed, whereas transfection with E7 and E2 siRNAs is accompanied by an increasing in TLR4 expression levels (56,57).

Immune system plays an important role determining or not the progression toward carcinoma (58). Besides the ability to induce cellular transformation, the proteins E6 and E7 have the property to interfere with innate and acquired immunity. Innate immunity is altered by E6 and E7 through interaction with the TLRs family, which recognizes molecular common motifs to many pathogens.

The E6 protein, also interacting with the interferon regulator factor3 (IRF-3), normally activated in response to a viral infection, inhibits the function of IRF-3 allowing the virus to revolve the antiviral response of the infected cell. In similar way, E6 protein also interferes on keratinocytes and Langerhans cells by reducing level of E-cadherin on the surface of keratinocytes, by limiting the presentation of viral antigen to the Langerhans cells, by promoting the persistent infection (59).

E7 is able to elude the immunological surveillance inactivating the functions of IRF-1 (60). It can also suppress the cytotoxic response, through the down-regulation of the transporter associated with antigen protein 1 (TAP-1), a key molecule in the mechanism of processing and antigen presentation. As result, there is a reduction of antigen presentation by major histocompatibility complex class 1 (MHC-1), making ineffective the response of cytotoxic T lymphocytes (CTL) (61). HPV also acts by altering the pattern of cytokine expression to revolve the immunological surveillance. The pathogenetic mechanism performed through the down-regulation of the expression of TNF- α , while the HPV E2 protein up-regulates the anti-inflammatory molecule IL-10, in order to limit the migration of non-resident immune cells towards the site of infection (62,63).

6. CONCLUSIONS

We have investigated about the possible relationship between TLR4 expression and HPV infection status in OSCC. ISH HPV positive samples have reported lower levels of TLR4 intensity than ISH HPV negative samples. Significant association between TLR4 expression and HR-HPV integration status and between TLR4 expression index and HR-HPV infection have been also observed. These interesting scientific results cast new light on HPV related and HPV-unrelated pathogenesis of oral cancer.

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Drs Giuseppe, Pannone and Pantaleo, Bufo, contributed equally to this work.

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Send correspondence to: Angelo Santoro, Department of Advanced Biochemical Sciences, Section of Anatomic Pathology, University of Napoli Federico II, Via S Pansini 5, Napoli, Italy, 10Department of Experimental and Clinical Medicine, Dental Clinic, University of Foggia, Foggia, Italy, Tel: 390817463433, Fax: 390817463414, E-mail: merollafr@gmail.com