PERI-TUMORAL INFLAMMATORY CELL INFILTRATION IN OSCC: A RELIABLE MARKER OF LOCAL RECURRENCE AND PROGNOSIS? AN INVESTIGATION USING ARTIFICIAL NEURAL NETWORKS

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The presence of inflammatory reaction in peri-tumoural connective tissue is generally considered as a defense mechanism against cancer, but inflammation tissue in malignant transformation and early steps of oncogenesis has been recently proven to play a supporting and aggravating role in some carcinomas. To evaluate in OSCCs the independent association of peri-tumoral inflammatory infiltrate (PTI) with local recurrence (LR) or survival outcome, and to verify whether PTI can be considered a marker of prognosis. Data from 211 cases of OSCC, only surgically treated between 1990 and 2000, were collected and retrospectively analyzed for PTI and the event LR (5 yrs follow-up at least) by means of univariate-multivariate and neural networks analyses. Patients (mean age 65.3 ± 12.4 yrs, M/F = 2.98) showed presence of PTI in 68.2% (144/211): (+) in 27.0%, (++) in 25.6%, (+++) 15.6%; PTI was found reduced in 24.7% of cases and absent in 7.1%. In overall PTI+ve group (n=144), 66 were TNM Stage I, 33 Stage II, 45 Stage III, none Stage IV. LR (mean 6 ± 4 months) was present in 87/211 (41.2%) patients, of which 43/144 (29.8%) in OSCCs with PTI [23 in PTI+ve (+), 13 in (++) and 7 in (+++)] vs. 44/67 (65.7%) in PTI -/+ or –ve ones. By univariate analysis, PTI+ve cases showed a significant lower risk to have LR (p<0.0001; OR= 0.2297; CI= 0.1277:0.4134) vs PTI -/+ or –ve ones, especially among cases with higher PTI value (+++) (OR= 0.1718; CI= 0.0749:0.3939). Multivariate analyses (Logit model and neural networks) confirmed the same datum: presence of PTI was an independent predictive variable accounting for a better tumoural outcome without LR (Logit and neural networks values: OR' 0.226; CI= 0.113:0.454; ROC Area = 0.66, respectively). In terms of prognostic significance, elevated PTI was found to have an independent association with the poorest overall survival rate (P = 0.056). Our findings strongly suggest the importance to investigate routinely PTI in OSCCs, as useful marker of tumoral behavior and prognosis, and warrant further studies on its specific cellular nature.

The presence of inflammatory cells in the peri-tumoral microenvironment is a common feature of many cancers, yet there remains significant controversy regarding its role with respect to tumor development, progression and spreading.

Peri-tumoral inflammation has been traditionally considered to represent a defense mechanism against cancer progression and invasion (1). Indeed the absence of tumor-associated lympocytes has been reported to represent a negative prognostic factor in a number of

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human malignancy including cutaneous melanoma, colorectal, breast, ovarian, and endometrial cancer (2).

Nonetheless, there is increasing evidence that peri-tumoral stromal inflammation is more likely to contribute to cancer development, growth and progression than mounting an effective host anti-cancer response (3). Several studies have highlighted that cancer cells can promote local migration of myelomonocytic cells which, in turn, can express inflammatory mediators supporting pro-tumoral functions such as cell proliferation and survival, angiogenesis, stroma remodeling and metastasis formation (4). Overall there remain significant controversy and unclear aspects upon the mechanisms that determine and orchestrate the differences between these two identities of peri-tumoral inflammation, namely the chronic smoldering inflammation promoting cancer-cell growth and survival versus the cytotoxic inflammatory reaction triggering cancer cell destruction (5).

Little is known regarding the role of the peri-tumoral inflammatory infiltrate (PTI) of oral squamous cell carcinoma (6), an aggressive epithelial malignancy that is characterized by a strong tendency to loco-regional spreading, lymph node metastases, local recurrences, and an overall poor prognosis. There remains little evidence suggesting whether or not the presence and the characteristics of PTI of oral squamous cell carcinoma (i) correlate with prognosis, (ii) could be used to predict clinical behavior and plan individualized treatment strategies (7). The aim of the present study was to investigate the role of PTI as independent prognostic factor for LR or survival outcome in a series of 211 patients with oral squamous cell carcinoma.

Data were studied using two statistical models: traditional univariate/multivariate analysis and artificial neural network (ANN). The advantages of ANN in detecting causal relationships among multiple factors that operates synergically and in correcting for multiple potential confounders has been reported before.

MATERIALS AND METHODS

Research aim and hypothesis

The aim of the present study was to investigate if the presence and grade of PTI could affect the incidence rate of LR and 5-year survival in patients with OSCC. The null hypothesis was that the LR incidence and 5-year survival rate would not vary regardless of the presence and grade of PTI

Study population

This was a retrospective study of histopathological specimens and clinical data relevant to a cohort of 211 patients who were diagnosed with oral squamosu cell carcinoma (OSCC) between 1990 and 2000 in the Sector of Maxillofacial Surgery (Politecnica delle Marche Ancona, Italy).

All patients received surgical resection of the primary tumor with curative intention, followed by cervical/submandibular lymphadenectomy and/or radiotherapy where indicated. Tumors were staged according to International Union Against Cancer (UICC) TNM system (8).

Data regarding primary tumor stage and initial treatment, as well as subsequent local recurrences, second primary tumors, lymph node or distant organ metastases, and related therapy for a period of at least 5 years were collected from clinical notes of all patients.

Histopathological analysis and PTI definition

Evaluation of histopathological specimens and slides was performed independently by two authors of the present paper (CR, TP) and include (i) OSCC diagnosis and grading, (ii) OSCC grading at deep invasive front and (iii) assessment and grading of PTI. Grading of PTI was performed on the basis of the number of inflammatory cells: (-) if PTI was absent, (-/+) if PTI was very mild, (+) if PTI was mild, (++) if PTI was moderate, and (+++) if PTI was intense. The analysis of PTI was determinated by T lymphocytes and B lymphocytes infiltration as described in Viera et al 2008 (9).

Prognostic outcomes and other variables

The following prognostic outcomes were considered: local recurrence, 5-year survival. Local recurrence (LR) was defined as tumor recurrence in the primary region after initial treatment, documented by surgical biopsy and histological evaluation. Other variables considered for data analysis included: tumor staging and grading, patient age and gender.

Univariate and multivariate statistical analysis

The data were analyzed by means of StatView for Windows (SAS Inc v. 5.0.1, Cary, NC, USA) and Statistica 6.0 (Statsoft Inc, Tulsa, OK, USA). The crude Odds Ratio (OR) and the 95% corresponding test-based Confidence Interval (CI) were calculated to measure the association level. The Chi-square test was used to assess statistical differences among categorical variables; p-values ≤ 0.05 for all evaluations were considered statistically significant. Regarding the criteria of acceptability, variables such as *age* and *tumor size* were categorized into nominal ones by utilizing the median values. Reference groups were chosen as follows: regarding an ordinal variable (e.g. *age*), the first category was chosen as the reference group; for other features, the category with the largest number was chosen as that reference group.

The relationship between LR occurrence and PTI, and the connection between survival outcome and other variables, was analyzed within univariate and multivariate frameworks. A logistic regression model was constructed, given the dichotomous nature of LR and outcome variables. A stepwise selection procedure was applied to obtain the most parsimonious model. The Maximum Likelihood Ratio (MLR) and Adjusted Odds Ratio (OR') were obtained using the iterative weighted least squares procedure. The 5-year survival rate was evaluated by the well-known Kaplan-Meier and Cox regression methods. The log-rank test was utilized for evaluating the differences in observed survival between groups. Regarding survival rate analysis, we categorized the deceased (due to OSCC) patients as

uncensored; the remaining patients were classified as *censored*.

Artificial neural network (ANN) analysis

Following a blinded procedure, three ANN models (see Fig. 1 for PTI *vs.* LR system) were applied to the same data; fully connected multilayer feedforward networks were also used. The learning rule was the well-known Back-error Propagation (BP) that adjusts the internal parameters of the networks over repeated training cycles to reduce the overall error (10). The networks were validated with a new set of data, different from the training ones, and the performance measured by: i) Mean Squared Error (MSE), Accuracy, Sensitivity and Specificity values; and ii) the Receiver Operating Characteristic (ROC) area, both effective at establishing the degree of meaningful prediction (11). Moreover, these indexes permit a comparison of performance between logistic regression and ANN systems. Regarding the latter, all variables were selected as input ones, except for LR presence, survival outcome and survival rate, which are all considered as output variables. No data variation is required because the ANNs can handle both numerical and nominal data.

RESULTS

The characteristics of the study population are reported in Table I, while details regarding features of sample grouped by LR are described in Table II.

ORs from PTI -/+ were found to be overlapping in the univariate preliminary analysis as compared to those of PTI –ve (p>0.2). Hence, we additionally labeled all cases PTI -/+ and –ve as *negative*. The main finding with univariate analysis was: overall PTI positive cases displayed a significantly lower risk (p< 0.0001; OR=0.2297 and 95% CI=0.1277:0.4134) of LR *vs.* negative PTI, especially cases with a higher PTI value (+++) (Table II). Even if other independent variables (site, tumor size, predominant grading, IFG and peri-neural infiltration) proved to be statistically significant risks for developing LR, PTI was identified as the most significant factor in the stepwise selection procedure (OR' 0.226; CI= 0.113:0.454). Specifically, Logistic Classification revealed an overall percentage of the correct estimate equal to 72.51%. Performance indexes (i. e. Methods) (LR +ve as *positive* and LR -ve as *negative*) were: accuracy = 72.51%, sensitivity = 60.92%, specificity = 80.65%.

Similarly, the ANN methodology proved that PTI was the most meaningful variable influencing LR in the present dataset, with an MSE equal to 0.43. Performance indexes possessed the following values: accuracy = 63.50%; sensitivity = 54.03%, and specificity = 77.01%. The ROC area was equal to 0.66.

As regards survival outcome, all results relating to univariate analysis are reported in Table III. The main result was that the most meaningful variables were all those which had been considered except *age*, *gender* and *site*, whereas, in multivariate analysis, it was found that

Table I. *Characteristics of study population*

Variables	no.	percentage (%)	mean (S.D*)	range
cases	211			
age (years)			65.3 (12.4)	28 - 95
sex Male Female	153 58	72.5 27.5		
histological grade G1 G2 G3	86 29 96	40.8 13.7 45.5		
invasive front grading G1 G2 G3	77 51 83	36.5 24.2 39.3		
tumor Size (cm) T1 T2 T3	104 74 33	49.3 35.1 15.6	2.23 (1.24)	0.6 - 6.0
regional lymph nodes N0 N1	167 44	79.1 20.9		
staging I II III IV	96 44 71 0	45.5 20.9 33.6 0		
oral site tongue gingiva mucosa sublingual	104 27 61 19	49.3 12.8 28.9 9.0		
perineural infiltration No Yes	112 99	53.1 46.9		
PTI score (-) (-/+) (+) (++) (+++)	15 52 57 54 33	7.1 24.7 27.0 25.6 15.6		
recurrence No Yes	124 87	58.8 41.2		

* *S.D= Standard Deviation*

only PTI and LR were identified as the best markers: PTI demonstrated a progressively protective role (OR'=0.430, CI=0.205:0.902), whilst LR was confirmed as a risk factor (OR'=4.867, CI=2.437:9.72). Considering the deceased patients as having *positive values* and the living

Table II. *Characteristics and risk factors grouped by Local Recurrences (LR)*

Univariate analysis					
	LR +ve (%) n=87 (41.2%)	LR -ve (%) n=124 (58.8%)	p-value	unadjusted OR OR(CI 95%)	
age at diagnosis, (years) *†<67 ≥67	44(43.1) 43(39.4)	58(56.9) 66(60.6)	#N. S	1.0 (reference) 0.8594 (0.4972 : 1.4855)	
gender *male female	65(41.5) 22(41.5)	93(58.9) 31(58.5)	#N. S.	1.0 (reference) 1.0153(0.5405:1.9074)	
site gingiva cheek mucosa *tongue oral floor	9(66.7) 28(45.9) 38(36.5) 12(63.2)	18(33.3) 33(54.1) 66(63.5) 7(36.8)	0.0298	0.8709 (0.3615:2.098) 1.4737 (0.7745:2.804) 1.0 (reference) 2.9874(1.1083:8.0519)	
tumor size (cm) *†<2 ≥2	31(32.6) 56(48.3)	64(67.4) 60(51.7)	0.0216	1.0 (reference) 1.9013 (1.0974:3.2944)	
predominant tumor histological grade *well(G1) moderate(G2) poor(G3)	23(26.7) 48(50.0) 16(55.2)	63(73.3) 48(50.0) 13(44.8)	0.0013 0.0052	1.0 (reference) 2.6434 (1.4581:4.7924) 3.5163 (1.451:8.5212)	
invasive front histological grade: *well(G1) moderate(G2) poor(G3)	9(17.7) 33(42.9) 45(54.2)	42(82.3) 48(57.1) 38(45.8)	0.0055 <0.0001	1.0 (reference) 3.1099 (1.468:6.5883) 4.5207 (2.2264:9.1796)	
Perineural infiltration *negative positive	36(32.1) 51(51.1)	76(67.9) 48(48.5)	0.0043	1.0 (reference) 2.216(1.2811:3.833)	
PTI *Negative Positive + ++ +++	44(66.7) 43(29.9) 23(40.4) 13(24.1) 7(21.2)	23(33.3) 101(70.1) 34(59.6) 41(75.9) 26(78.8)	<0.0001	1.0 (reference) 0.2297 (0.1277:0.4134) 0.3638 (0.1796:0.7368) 0.191 (0.0934:0.3904) 0.1718 (0.0749:0.3939)	
TNM Stage *I II III	29(30.2) 23(52.3) 35(49.3)	67(69.8) 21 (47.7) 36(50.7)	0.0121	1.0 (reference) 2.5557(1.2244:5.3343) 2.2316 (1.1895:4.1864)	
primary tumor size(T) *1 2 3	32(30.8) 40(54.1) 15(45.5)	72(69.2) 34(45.9) 18(54.5)	0.0018	1.0 (reference) 2.6149(1.427:4.7916) 1.9095 (0.8394:4.3437)	
regional lymph nodes (N) *0 1	64(38.3) 23(52.3)	103(61.7) 21(47.7)	#N. S.	1.0 (reference) 1.7735 (0.9047:3.4767)	
Multivariate analysis §					
	OR' 95%CI upper limit				95%CI lower limit
PTI	0.226 0.454				0.113

*Reference group for OR calculation. For details, see Materials and Methods
†Median value in comprehensive sample
#Not significant

Table III. Characteristics and risk factors for survival outcome

Univariate analysis				
	alive (%) n=107 (50.7%)	deceased (%) n=104 (49.3%)	p-value	unadjusted OR OR(CI 95%)
age at diagnosis, y *†<67 ≥67	56(54.9) 51(46.8)	46(45.1) 58(53.2)	#N. S	1.0 (reference) 1.3813 (0.8059:2.3675)
gender *male female	82(51.9) 25(47.2)	76(48.1) 28(52.8)	#N. S	1.0 (reference) 1.2072(0.6489:2.246)
site gingiva cheek mucosa *tongue oral floor	13(48.1) 30(49.2) 58(55.8) 6(31.6)	14(51.9) 31(50.8) 46(44.2) 13(68.4)	#N. S	1.3562 (0.5817:3.1617) 1.301 (0.6917:2.4469) 1.0 (reference) 2.6152(0.9866:6.9321)
tumor size (cm) *†<2 ≥2	58(61.1) 49(42.2)	37(38.9) 67(57.8)	0.0065	1.0 (reference) 2.115 (1.231:3.6336)
predominant tumor histological grade well(G1) *moderate(G2) poor(G3)	52(60.5) 44(45.8) 11(37.9)	34(39.5) 52(54.2) 18(62.1)	0.0484	0.5578 (0.3119:0.9975) 1.0 (reference) 1.3746 (0.5974:3.1628)
invasive front histological grade well(G1) moderate(G2) *poor(G3)	35(68.6) 39(50.7) 33(39.8)	16(31.4) 38(49.3) 50(60.2)	0.0012	0.3178 (0.1586:0.6367) 0.6458 (0.3469:1.2022) 1.0 (reference)
Perineural infiltration *No Yes	67(59.8) 40(40.4)	45(40.2) 59(59.6)	0.0049	1.0 (reference) 2.1666(1.2632:3.7162)
PTI (2° analysis) *Negative Positive	20(29.9) 87(60.4)	47(70.1) 57(39.6)	<0.0001	1.0 (reference) 0.2961 (0.166:0.528)
TNM Stage *I II III	59(61.5) 20(45.5) 28(39.4)	37(38.5) 24(54.5) 43(60.5)	0.0049	1.0 (reference) 1.9084(0.9317:3.9091) 2.404 (1.3032:4.4345)
primary tumor size(T) *1 2 3	63(60.6) 30(40.5) 14(42.4)	41(39.4) 44(59.5) 19(57.6)	0.0083	1.0 (reference) 2.2224(1.2257:4.0295) 2.0794 (0.9471:4.5653)
regional lymph nodes (N) *0 1	91(54.5) 16(36.4)	76(45.5) 28(63.6)	0.034	1.0 (reference) 2.0581 (1.0608:3.9931)
recurrence *No Yes	85(68.5) 22(25.3)	39 (31.5) 67(74.7)	<0.0001	1.0 (reference) 5.7258 (3.3256:9.8581)
Multivariate analysis §				
	OR'	95%CI lower limit	95%CI upper limit	MLR test p value
PTI **recurrence**	0.430 4.867	0.205 2.437	0.902 9.72	0.00249 <0.0001

Reference group for OR calculation. For details see Materials and Methods
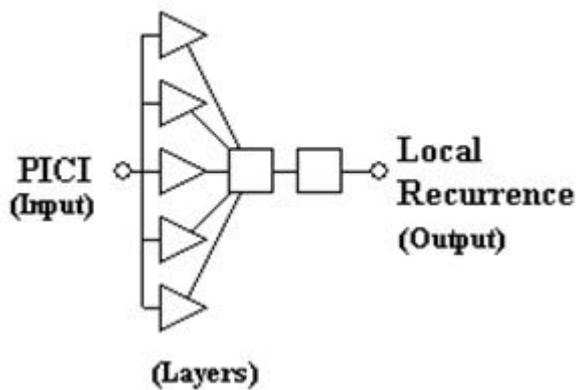*†Median value in comprehensive sample*
#Not significant

Fig. 1. *ANN structure applied in the study*

as *negative*, accuracy was 73.93%, sensitivity 69.23% and specificity 78.50%.

When applied to the same outcome problem, the ANN system performed well with an MSE equal to 0.3137; the area under the ROC curve was 0.8746. The meaningful variables were PTI, LR, IFG and staging (accuracy = 78.56%, sensitivity = 67.74%, specificity = 90.59%).

The analysis of the 5-year survival rate revealed that the highest mean lifetime was associated with PTI +ve cases and in LR-ve patients (p-value log-rank test < 0.0001)(Fig 2). Moreover, utilizing the Cox method, the LR variable proved to be the most meaningful (MLR p <0.001, OR' = 0.206). ANN performance was good (RMS 5.36) and the selected variables, in order of importance, were: LR, stage, PTI, peri-neural infiltration, IFG, predominant grading.

We also looked for secondary relationships among the survival outcome, survival rate and all of the

variables considered, regardless of LR, by repeating the aforementioned analyses. With respect to survival outcome, the PTI variable proved to be the only significant variable in the logistic analysis (p-value 0.0001 by MLR, 95% CI = 0.077:0.416; accuracy = 75.42%; sensitivity = 62.50%, specificity = 84.11%), while the most meaningful variables of the survival rate were: PTI (OR' = 1.808), IFG (OR' = 0.415), peri-neural infiltration (OR'=0.894), T (OR' = 0.558), N (OR' = 0.660).

DISCUSSION

The overall incidence of OSCC, the most frequent malignant tumor of the oral cavity, has progressively increased in recent decades. Paradoxically, in spite of an improvement in therapeutic procedures, it shows a generally poor prognosis due to its local aggressiveness and metastases either in young than in old individuals (12).

As PTI has been considered a defense mechanism against cancer in several types of human neoplasia (13), the ability of PTI to influence the Odds Ratio of LR on OSCC was investigated in this study, using traditional statistics and ANN system analyses. The latter revealed that the presence of PTI acts as an independent predictive variable accounting for a better tumoral outcome without LR.

The importance of host response against tumor cells has been observed in several previous reports (14-16)

Having proven the protective effect of PTI in OSCC outcome, our study investigated: the influence of PTI in 1) LR rate and the 2) survival outcome in patients affected by OSCC. Regarding survival rate and 5-year outcome, the statistical tools demonstrated that the most meaningful variables were PTI and LR, as also confirmed by ANNs. As expected, patients with LR had a poorer survival
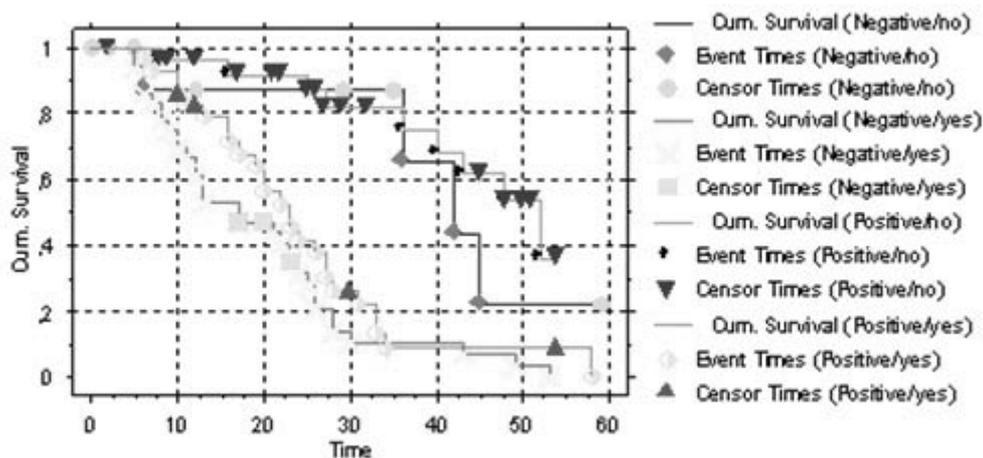

Fig. 2. *Kaplan-Meier survival plot, according to PTI grouping (negative/positive) and LR stratification (yes/no)*

rate than subjects without LR and, consistent with our aforementioned findings, cases with PTI had a better survival rate than cases without PTI.

In this study, in which we investigated only inflammation infiltrate in peri-tumoral connective tissue, the flogistic response has demonstrated a protective effect against tumors: the inflammatory cells in peri-tumoral connective tissue may well represent a mature 'defence line' in opposition to the 'enemy'. Conversely, inflammatory cells infiltrating the tumor could be considered as the 'front-line troops' which, due to a lack of maturation or proximity (e.g. the paracrine mechanism), are 'taken prisoner' and recruited by neoplastic cytokines cells. Our understanding of the mechanisms of cytokines and chemokines is inchoate and greater efforts will be required to elucidate the relationship between neoplastic and inflammatory cells.

To the best of our knowledge, ours is the first report in which Artificial Neural Network system (ANNs) have been used in the largest sample size of OSCC patients to investigate which clinico-pathological variables could exert an influence on biological behavior and long-term outcomes in OSCC. ANNs are non-linear regression computational tools which have been used in classification and survival prediction in several biomedical systems. They are able to detect non-linearities among variables relating to interest, learning and adaptability, and they possess robustness and noise tolerance (17).

In conclusion, the null hypotheses considered as our primary endpoints were verified as PTI expression was found to be a reliable inverse marker of LR and a positive prognostic factor in terms of survival outcome and rate. This suggests the importance of investigating PTI-specific cellular nature and to routinely survey PTI in OSCC. Moreover, taking in account this aspect, the role of PTI can be further investigated in other age-related cancer as for example prostatic cancer. To conclude, in our study, ANNs has showed to perform better with respect to the traditional statistics for survival outcome analyses, warranting their ever-increasing use in this field of clinical oncology.

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