Author's response to reviews

Title: Prognostic value of p27Kip1 expression in Basaloid Squamous Cell Carcinoma of the larynx.

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Author's response to reviews: see over
Answer to Reviewer’s reports

Giorgio Cortesina

a) Statistical analysis

Discriminant analysis (D.A.) begins with the desire to statistically distinguish between two or more groups of cases.
To distinguish between the groups we selected a collection of discriminating variables that measure characteristics on which the groups are expected to differ. D.A. attempts to do this by forming one or more linear combinations of the discriminating variables.
The statistical theory of D.A. assumes that the discriminating variables have a multivariate normal distribution and that they have equal variance-covariance matrices within each group. In practice, the technique is very robust and these assumptions need not be strongly adhered to.
Once the discriminant functions have been derived, we are able to pursue the two research objectives of this technique: analysis and classification.
The stepwise method was used. Independent variables were selected for entry into the analysis on the basis of their discriminating power. In many instances the full set of independent variables contains excess information about the group differences, or perhaps some of the variables may not be very useful in discriminating among the groups. By sequentially selecting the “next best” discriminator at each step, a reduced set of variables will be found.
We use the method named as WILKS. The stepwise selection criterion used was the Wilks method. The criterion is the overall multivariate F ratio for the test of differences among the group centroids. The variable which maximizes the F ratio also minimizes Wilks’ lambda, a measure of group discrimination. This test takes into consideration the differences between all the centroids and the cohesion (homogeneity) within the groups.
The use of a stepwise procedure results in an optimal set of variables being selected. The assumption is that stepwise procedure is an efficient way of approximately locating the best set of discriminating variables. Here, two groups were considered: cases having a value of 1 on the variable group (i.e., alive) were considered to be members of group 1, while cases having a value 2 on the variable group (i.e., not alive) were considered to be members of group 2.
Finally, remember that the significance level in this case is the probability of obtaining differences in the centroids as large or larger than are found in this data due to chance when the centroids are actually equal in the population. As the centroids become more separate (i.e., F gets bigger) the associated significance level \( p \) becomes smaller.
Any way, the significance of the variable (P27), selected at the first step, was \( p=0.01 \).

The value of significance used was 0.05

b) Immunochemistry section

Bibliographic references were added (see text)

Tim Helliwell

Methods:

The authors need to give more detail on the methods of assessment of the immunocytochemical staining:

what area of tumour was evaluated? **5 different fields of represented areas of tumors.**

what magnification was used? **The magnification was x100**

what criteria were used to determine positivity or negativity for each cell? **We evaluated the nuclear, cytoplasmic and membranous positivity**

how did the pattern of staining vary in different parts of the tumour, and how was this allowed for in the assessment? **No variation concerning the pattern of staining has been found in all the tumors evaluated.**

how much variation was there between observers? **Negligible variation**

how were the cut-off points for high and low expression determined? if these are arbitrary cut-off points, how critical is the precise % value in influencing the results? **See References**


In the results section the authors should:

1. provide raw data with median % score and range for each antibody and each case. **See text, Tab.II**

2. the second paragraph of the results should be moved to later in the results after the data have been presented. **OK**

3. the second half of the fourth paragraph (description of immuno work) should be moved to the end of the second paragraph - some description of intratumoural variability would be useful here. **No intratumoral variability has been found**
4. data should be presented (median, range) on p53 and Ki67 results, as they have been used in the analysis but are not presented. **OK, see text and statistical tables for each cases (16)***

In the discussion:

1. there is mention that Ki67 and p53 stained more intensely and diffusely in DOD patients – this does not appear in the results and there is no mention of its statistical significance.

The immunohistochemical study showed that p27\textsuperscript{kip1} was highly expressed in 40% of the NED patients and, strikingly, in none (0%) of the DOD patients, whilst p53 and Ki67 were highly expressed respectively in 60% and 80% of the patients in NED status and in 90% and 100% of the patients in DOD status (Table III). At multivariate analysis high p27\textsuperscript{kip1} expression demonstrated to be significant (p=0.01). No significant inverse correlation was found between p27\textsuperscript{kip1} expression and p53 or to Ki67/Mib-1 expression in DOD and NED patients groups. When the clinical series was globally evaluated, a slight trend for inverse correlation (P=-0.33 for p53 and P= -0.447 for Ki67/Mib-1) resulted.

2. there is mention of an inverse correlation between p27 and p53 and Ki67 - no details are given in the results. **See previous answer**

3. there is no discussion of the potential prognostic relevance of p53 and Ki67 results.

In our series, p53 and Ki67 were globally highly expressed both in the patients with NED and in the DOD group, with a slightly lower expression in the former samples compared to the DOD patients. The finding that both Ki67 and p53 are over-expressed in a relevant fraction of our patients exhibiting poor outcome is in agreement with numerous published studies attributing to the above proteins a role as predictors of adverse prognosis in several human tumour types, including head and neck cancers. Never less, Ki67 and p53 expression were widely and abundantly retained also in patients in NED status, and were not able to discriminate, in a significant manner, between different outcomes. The result is not surprising in itself, considering the high proliferative capability and potential of aggressiveness of BSCC when compared to different histotypes. By the way, the potential prognostic relevance of p53 and Ki67 was not the aim of the present study, focused on the possible prognostic relevance of the evaluation of p27 protein expression. In facts, our aim was to demonstrate that the downregulation of p27 constitutes a hallmark of biological aggressiveness of BSCC.