Morpholiogical, quantitative image analysis and molecular biology of oesophageal squamous cell carcinoma (ESCC), with special reference to tumour progression and human papillomavirus (HPV) involvement

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Abstract

Background: It is an urgent need to improve early diagnosis of ESCC, beacauses is one of most agressive cancer. The quantitative image analysis, can aid in the identification of ESCC. Despite much research effort, the major prognostic factor of ESCC remains the pathological stage of the disease as defined by the TNM classification, whereas tumour grading is of limited value in this respect, mainly due to its low reproducibility. A better means for disease prognostication based on improved understanding of the pathogenetic mechanisms is urgently required.
Materials and methods: The material of the present study was derived from a series of 1876 oesophageal surgical specimens taken from a total of 700 patients, who underwent oesophageal resection for an invasive ESCC in Anyang Tumour Hospital, Henan Province of China. Among. The cases of ESCC, previously subjected to extensive testing for Human Papillomavirus (HPV) involvement and expression of p53 gene. All cases are analysed by histopathology and by in situ hybridisation (ISH) and PCR, and a group of 272 patients was randomly selected for analysis of the primary tumour, adjacent mucosa and regional lymph nodes, in the quantitative image analysis. All cases and HPV data were subjected to extensive univariate and multivariate analysis to disclose independent predictors of progressive disease.

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Results: For the analyses, the ESCCs were graded into two categories: well - moderately and poorly-differentiated. HPV DNA was detected in 116 (18.9 %) of the carcinomas by ISH and in 15.2 % by PCR. In univariate analysis, lymph node status was significantly ($p < 0.01$) predicted by the following nuclear parameters: nuclear area, G0/G1 ratio, HPV DNA status, integrated optical density (IOD), mean optical density (MOD) and S-Phase. In multivariate analysis, 6 variables remained as independent predictors of disease progression ($p < 0.05$ level), the three most significant ones being nuclear perimeter, nuclear roundness and equivalent diameter ($p < 0.01$).
Conclusions: A series of quantitatively measured nuclear parameters seem to be a close correlation with ESCC differentiation and progression in univariate analysis and some of these variables proved to be significant independent predictors of disease progression in multivariate modelling as well. These data clearly advocate the use of quantitative image analysis in searching for additional prognostic factors of ESCC.

Key Words: *Oesophageal squamous cell carcinoma, prognosis, quantitative image analysis, diploidy, Human Papilloma Virus, hybridisation in situ, PCR.*

Introduction

It is an urgent global need to improve early diagnosis of ESCC, beacauses one of most agressive cancer. In the 2018, by International Agency for Research on Cancer (IARC), the oesophageal cancer is diagnosed in 572 034 new cases, and 508 585 deaths in one year. Its worldwide incidence is to be increased. [1, 2] About 60 % of cases are ESCC, and 30 % adenocarcinoma. [1, 2, 3, 4]

ESCC represents a leading cause of cancer mortalty in certain geographical regions, as Northern China, South Africa, Costa Rica, Afganistan and Iran, but is a relatively uncommon neoplasia in Western countrises. The five-year survival rate of ESCC in the USA is 19 %, primarly beacause is often at an advanced, incurable stage, while and in Golestan province of Iran it is 3, 4 %. [4, 5, 6, 7]

The geographical distribuation of ESCC, clearly implicate a dominant role for environmental factors in the etiology of this disease. [4, 5, 6] A wide variety of such factors have been studied as potential etiological agents, including cigarette smoking (with 35 cancerogen substances), alcohol, exposure to nistrosamines and mycotoxins in the aliments, opium use, nutritional deficiences and hot beverages. [3, 4, 5]

The carcinogenesis of ESCC is a multifactorial and multistep process. It involves various genetic and environmental factors. A number of risk factors have been studied as potential etiological agents, including cigarette smoking (contains

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35 cancerogen substances), alcohol, exposure to nistrosamines and mycotoxins in the aliments, opium use, nutritional deficiences and hot beverages. [3, 4, 5]

Apart from the large-scale epidemiological surveys, molecular pathogenesis hes been studied as well, with several ocogenes, tumour suppressor genes, and infectious agents, particulary the role of HPV infections, is implicates in the pathogenesis of ESCC in the high incidence areas.

Despite improved detection methods and therapy, the prognosis of this neoplasia remains ominous, and there is an imminent need for a better understanding of the biology, the behavior of ESCC, and its prognostication. For this purpose, a wide variety of factors have been analysed to predict the clinical course of ESCC, including cell ploidy, cell proliferation markers, cellcycle markers, oncogenes, tumour suppressor genes, growth factors, invasion patterns, tumour-matrix interaction, growth factors, adhesion molecules, immunological factors, histopathologically parameters, mytotic index, lymph node involvement, tumour stge, biological factors, prognostic significance of quantitatively measured cell variables in ESCC and the expression of different cytokeratin molecules as potential marker of tumour differentation. [5, 7, 8, 9, 10, 11] However, the major prognostic factor of ESCC remains the pathological stage of disease as defined by the TNM classification, with the WHO grading and pT classification. [4]

The key to undertsanding the clinical behaviour and the prognosis of ESCC is the uncoverting of the pathogenetic mechanisms resposabile for the developement of this disease through its precursor lesions: epithelial metaplasia and dysplasia. [10] As result of this study, is confirmed the frequent involvement of HPV, also the potential role of CK18 expression in progressive disease, and mutations in the p53 gene, which is co-existent with HPV infections. [4, 5, 6, 7, 8, 9, 10, 11]

The present sudy was conduced to analyse a wide variety of quantitaively measured nuclear variables of the cancer cells and their relationship to disease progression. These parameters and other availabel data were subjected to extensive univariate and multivariate analysis to assess their role as predictors of disease progression in this series of ESCC.

Material and methods of the study

***Patients and samples*: **The material of this study was derived from e serie of 1876 oesophageal surgical specimens taken from a total of 700 patients, who underwent oesophageal resection for an invasive ESCC in Anyang Tumor Hospital, in Henan Province of China. All patients are from Linxian – the high risk area for ESCC in Northern China, where the age – adjusted mortality rates for this tumor are 161 males for 100 000 habitans, and 103 females for 100 000 habitans. [1, 2, 5] From

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this coohort, cancer samples of 273 patients were subjected to quantitative image analysis and citometry, fro which 57 % males and 43 % females. The age of the patients ranged from 36 to 72 years, with a mean age 54, 8 years (SD 8, 7) in the males, and 55, 5 years (SD 8, 6) in the females. From each patients, surgical specimens were taken from a. the primary tumour; b. adjacent oesophageal mucosa; c, regional lymph nodes, d. as well as surgical resection margins.

Histopathology. Routinly processed, haematoxylin – eosine – stained sections were examined with light microscopy to confirm:

- The diagnosis of ESCC, to grade the intraepithelial lesions adjacent to carcinoma, (absent, mild, moderate, severe dysplasia - Intraepithelial Neoplasia of Oesophagus).
- To conferm or exclude the lympho node metastases.
- To valuate the morphological evidence for HPV involvement, using the criteria by Syrjänen.

In positive cases, the HPV suggestive morphology was classified as papillary, flat or inverted. [6, 7, 8]

Histological grading of ESCC into three degrees (well, moderately, poorly differentiated), was initially performed by three pathologists based on criteria: a. nuclear polymorphism; b. degree of keratinisation, and c. mitotic activity.

HPV screening in Situ Hybridisation**:** All biopsies were analysed to HPV-DNA detection by screening in ISH, with biotn-labelled HPV cocktail probes (detecting a wide spectrum of HPV), under low-stringency conditions (Tm-35), as detailed previously. [5, 6, 7, 8] the hybridisation in situ signals were developed with nitroblue tetrazolium and bromo – chloro – indoxyl phosphate at 37° C for 2 hours.

HPV type specification in Situ Hybridisation: The cases HPV DNA positive samples on the ISH screening were further analysed by HPV type specific ISH using Biotin-labelled HPV DNA probes under highstringency conditions (Tm-17). The technique has been detailed before [5, 6. 7. 8]. For HPV typing, the whole genomic DNA probes of HPV 6, 8, 11, 16, 18, 18, 33 and 53, were biotinylated by nick-translation using a commercial kit, at the concentration of 1.0 mg/ml in the hybridization cocktail. CasKi cell smears, and formaline - fixed, paraffine - embedded biopsies which had previously been shown to contain HPV DNA sequences were used as positive controls in both the screening and type specific ISH. [5, 8, 10, 12]

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***Detection of HPV DNA by polymerase chain reaction (PCR)*:** The presence of HPV DNA in the biopsies of our study was analysed with PCR using MY09/MY11L1 consensus primer sets: 5'-CGTV CC (A/C) A(A/G)(A/G)GGA (A/T)AC TGA TC-3', and 5'GC(A/C) CAG GG(A/T) CATAA(C/T) AAT GG3' All samples were tested initially using β-globin gene specific primers (5'-ACA CM CTG TGT TCA CTA GC-3', and 5'-CAA CTT CAT CCA CGT TCA CC-3'), which amlify a 100-bp region of β-globin gene. PCR was performed as previously described in detail from Villiers. (8, 12) A total of 35 cycles of amplification of the target sequences were carried out in 50 ml of a reaction mixture with the Gene Ampli Taq kit and the Perkin Elmer Cetus automated thermal cycler. The specific HPV DNA sequences amplified by the PCR were subsequently confirmed by Southern Blot Hybridization, with P-labelled HPV 6, 11, 16 and 18 DNA probes, under low-stringency conditions (Tim-35). [4, 5, 9, 10, 12]

Image analysis: For quantitative image analysis, 6 μm thick formalin-fixed, paraffine-embedded tissue sections were used, stained for DNA according to the Feulgen procedure.

The Feulgen stained histological slides were analysed using the LEICA Q500MC image analysis system (LEICA England), coupled with true colour CCD video camera (JVC – Japan) and Leica microscope.

The image cytometry was performed according to the consensus Report of the European Society of Analytical Cellular Pathology. [4, 10]

Microscopic fields (4 to 6), under visual control, were chosen from multiple areas representative of the tumour sections, according to the following criteria: a. completeness of the cellular nucleus, assessed by focusing up and down; b. no over, or under lapping of neighbouring cellular nuclei; c. assertion that nuclei belong to the tumour cell popullation. [4, 10]

Each microscopic field was stored at 512 x 512 pixel with 256 grey levels and measured using the Qwin's software (Leica). Apporximately 150 to 300 cellular nuclei per field, with a total at least 450 per specimen, excluding lympocytes, macrophages and fibrobllastes. The following parameters were measured:

A. *Morphometric*: 1. nuclear area, 2. perimeter, 3. length, 4. breadth, 5. convex perimeter, 6. roundness, 7. curve length, 8. curve width, 9. aspect ratio, 10. convex area, 11 equivalent diameter.
B. *Densimetric*: 1. Mean Optical Density (MOD), 2. Integrated Optical Density (IOD), 3. Gray Variance. The DNA index (DI) was calculated as the ratio of the mean channel value of the G0-G1 peaks of the tumour cell population to that of the G0-G1 peak of dipolid reference. The position fo diploid channel was verified by examining the peak channel number produced by bening

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lymphocytes that were present in each cases, so we are used as 100 – 150 lymphocites as internal quality control.

In DNA – IMC, the mean channel value of the G0-G1 peak of the tumour nuclei was 24 whereas that of lymphocites was 20, 8; the coresponding DNA index was 1, 14. Because of the small value of shift, we did not use any correction factor. [4, 10]

A diplod tumour peak was defined as a single peak with a DI = 1. 00 ± 2 SD (1 ± 0, 15), and non other peaks more 10 % of the total were present.

A aneuploid peaks was defined by the presence of a peak > 10 % of the total lying outside the diploid range, with a DI 1, 2 – 2, 0.

Tetraploid peaks were defined as a G2/M peak (4N) > 15 %, with a DI between 1, 85 – 2, 15.

Statistical analysis: Were performed using the SPSS computer program peckage (version 6. 1).

Frequence tables were analysed using Chi-square test, interpreting the Ch-square statistics, Fisher's exact test, Pearson's R, Spearman correlations and/or likelihood ratio were used to assess the significance of the correlation between the individual variables in unvariate analysis.

Difference in the means of the morphometric vairables between the groups (Node +/Node –; and Tumour Grade 1/Grade 2); were analysed using the analysis of variance (ANOVA) models, with F statistics. The correletaion of different quantitative nuclear parametres to 1. Lympho node status, 2. HPV status and 3. Tumour Grade, were tested bu Mann-Whitney U test. Wilcoxon Rank sum W test, Kolmogorov – Smirnov 3 – sample test and/or by Kruskla – Wallis 1-way ANOVA test.

Multiple logistic multivariate models were used to analyse the indipendent prognostic value, of variables, using the stepwise backward approach. Removal testing was based on the probability of the likelihood ratio statistic. Both morphological variables shown to be significant in the univariate analyses were entered into the model, with p = 0. 10 being the probability for stepwise removal and p = 0. 05 the probability for stepwise entry.

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Results

Histopathological analyses and HPV detection. Results of the histopathological analyses are included in Table 1, where the numbers of the total series and the caseses subject to image analyses are shown seprataly.

Chracteristics	Number of cases		per cent	
	Morphometry series	Total series	Morphometry series	Total series
Grade of ESCC				
well differntiated	105	198	38. 5	28. 3
moderately differentiated	89	357	32. 7	51. 0
poorly differentiated	79	145	28. 8	20. 7
Ploidy				
Diploid	220		80. 2	
Non diploid	53		19. 8	
Lympho node metastses				
Present	40	145	46. 5	46. 4
Absent	46	167	53. 5	53. 6
Non information	187	388		
Adjcent tissue present	152	538	55. 7	76. 8
Dysplasia in ajacent tissue				
No	101	246		
Mild	7	21	4. 6	5. 7
Moderate	15	31	9. 9	8. 4
Severe	29	69	19. 1	18. 8
HPV – suggestive changes in adjacent tissue				
No	129	322	85. 4	88. 2
Papillary	2	5	1. 3	1.4
Flat	20	38	13. 2	10. 4
HPV – suggestive changes in tumor tissue				
No	139	387	67. 1	69. 7
Papillary	24	69	11. 6	12. 4
Flat	11	16	5. 3	2. 9
Endophytic	33	83	15. 9	15. 0
HPV detectetion by ISH				
Positive	49	116	22. 5	18. 9
HPV detection by PCR				
Positive	3	66	1. 4	15. 2

From 700 carcinomas, 198 cases (28, 3 %) were well differentiated, 357 cases (51. 0 %) as moderately differentiated and 145 cases (20. 7 %) as poorly differetiated. For the final analyses, well and modertaely differentiated carcinomas, were combined as Grade 1, and poorly differentiated as Grade 2.

Regional lymph node metastases were indentified in 145 cases (46. 5 %) of the 312 pateints.

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All grades of dysplasia were observed in the adjacent tissues, albeit in the majority (33. 0 %) of the availabel cases. Adjacent tissues also combined chenges suggestive of HPV in a minority of caeses (11. 8 %)

Such HPV – suggestive morphological changes were detectable in 30. 3 % of the primary tumours, covering all morphological variants of HPV (papillary, flat and inverted). HPV DNA was detected in 116 (18. 9 %) of carcinomas. PCR positivity was of the same order of magnitude 15. 2 % of the cases analysed. The agrement between ISH and PCR was calculated with the kappa – statistics (kappa 0. 5803).

Results of image analysis. 273 cases (about 40 % of all ESCC) was examined to image analysis, using morphometry and densitometry. Table Nr. 2 gives the descriptive statistics of the parameters analysed. All these morphological variables were subjected to analyses by ANOVA models and non-parametric tests, to compare the means between the groups Node + / Node – cases, and to correlate these variables to lympho node status, also HPV status and tumour grade, respectively. In ANOVA tests, no statistically significant differences between Node + / Node – cases were disclosed in any of these parameters.

No significant correlations between the tested variables and three grouping variables could be found by the non – parametric test either. The DNA index (DI) was calculated as the ratio of the mean channel value of the G0-G1 peaks of the tumour cell population to that of the G0-G1 peak of dipolid reference. DNA index was 1, 14. [4, 10]

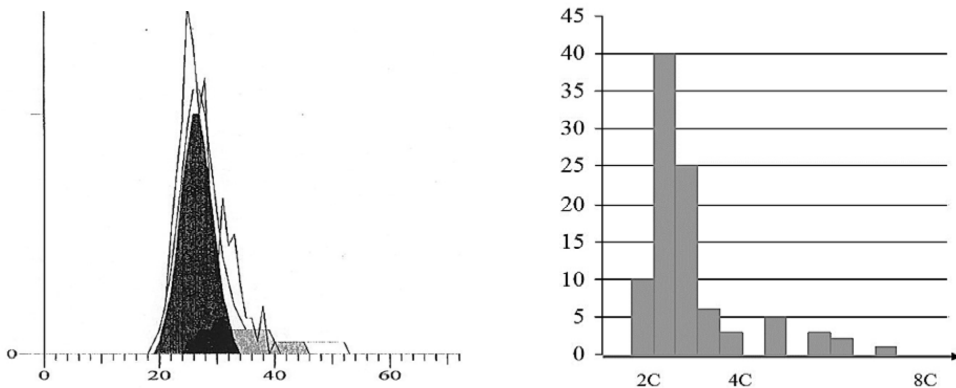

FIG. 1. Polynomial curve and histogram of DNA diploidy.

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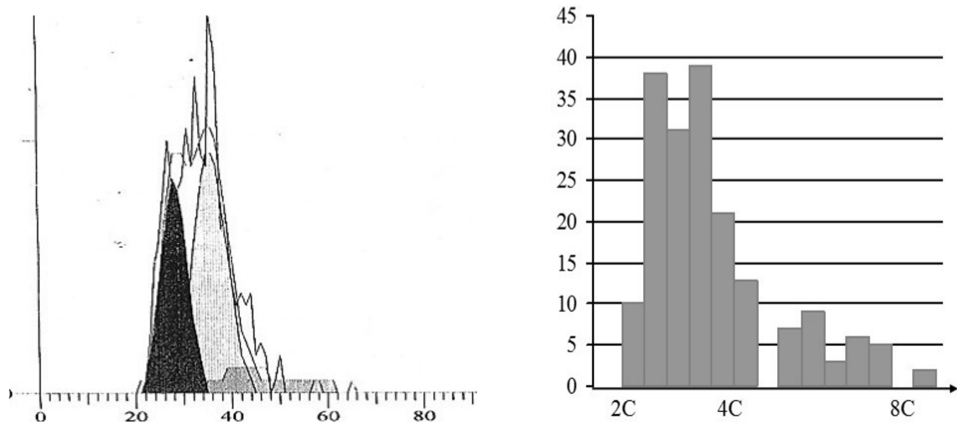

FIG. 2. Polynomial curve and histogram of DNA aneuploidy.

For all cases analysed in static cytometry were make the polynominal curve and histiogram of DNA ploidy. - 52 cases (19,05 %) of ESCC are DNA diploidy (p = 0. 1) (Fig. 1)

A diplod tumour peak was a single peak with a DI = 1. 00, SD (1 ± 0, 15), and non other peaks

> 10 % of the total were present.

- 221 cases (80, 95 %) of ESCC are DNA aneuploidy (p = 0. 12) (Fig. 2) A aneuploid peaks was with the presence of a peak > 10 % of the total lying outside the diploid range, with a DI 1, 2 – 2, 0.

Tetraploid peaks were defined as a G2M peak (4N) > 15 %, with a DI between 1, 85 – 2, 15.

- Cells fraction in S – phase, from both ESCC dyploidy and ESCC aneuploidy were 32, 4 % (SD +/- 18, 3).

- Cells fraction in phase G2 - M were 2,12 % (p = 0. 02)

- Cells fraction in phase G0 - G1 were 66, 07 % (p = 0. 02)

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TABLE 2. Statistics of the parameters analysed by image analysis.

Parameters	N° cases	Mean	Median	SD
Index DNA	272	1, 247	1, 210	0, 769
G0-G1	272	66, 068	67, 010	20, 265
G2-M	272	2, 122	1, 370	2, 466
S - phase	261	32, 386	31, 160	18, 237
Grey variance	132	0, 014	0, 014	0, 040
IOD	272	111718, 905	110565, 000	20292, 141
MOD	273	0, 615	0, 609	0, 088
Nuclear area	273	48010, 000	47148, 000	6123, 433
Aspect ratio	273	1468, 703	1454, 000	124, 508
Breadth	273	6618, 956	6571, 000	489, 101
Convex area	273	53150, 447	51908, 000	7244, 374
Convex perimeter	273	25934, 817	25723, 000	1740, 177
Curve length	273	26765, 000	26765, 000	681, 836
Curve width	273	6533, 721	6550, 000	673, 416
Equivalent diameter	273	7747, 381	7689, 000	470, 829
Fullnes Ratio	132	0, 950	0, 952	0, 017
Length	272	9585, 756	9484, 000	743, 680
Perimeter	273	27401, 465	27164, 000	1787, 721
Roudness	273	1191, 956	1189, 000	49, 704

Univariate analysis. The variables analysed were tested fr their significance as predictors of the lympho node status, HPV detection and tumour grade, using Chi-square test.

Lympho node status was significantly ($p = 0.01$) predicted by a number of nuclear variables: nuclear area, G0/G1 ratio, HPV DNA status, IOD, MOD and S-phase and with borderline significance ($p < 0.05$), also by the DNA – Index, G2/M, nuclear length, perimeter and roudness.

HPV status was predicted by full ratio, MOD and roudness ($p < 0.05$)

The grade of carcinoma was higly significantly associated with HPV – suggestive morfological changes ($p > 0.000001$) and significantly with MOD and nuclear roudness ($p< 0.01$)

Multivariate analysis. All variables show to be significant in univariate analysis were entered into the multiple logistic regression analysis, using the stepwise likelihood ratio model.

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The three most significant ones ($p < 0.001$) are nuclear perimeter, nuclear roudness and its equivalent diamater. As the last step, all variables without significance in the univariate models, were individually entered into this "saturated" model.

Then, two additional factors proved to be indipendent, i. e, adjacent dysplasia and breath of the nucleus, both with level $p < 0.05$. In that case, nuclear breadth replaced nuclear length in the model.

Discussion

The present study was a part of our multinational EC-funded project, strated in the early 1990 in collaboration with Europiean and Chinese authors to elucidate the role of HPV in oesophageal ccarcinogenesis. [4, 5, 6, 7] Esophageal cancer is among the top ten most frequent malignancies worldwide, also in China remins one of the most common malignancies and has a high metastasis rate and poor prognosis. [1, 2, 4, 5]

It's generally agreed that the most significant prognostic oarameters are the pathological stage of the ESCCC as defined by the TNM classification. [3, 5, 6, 7, 8]. Recently, attemps have been made to develope a varety of prognostic scores, shown to favourable compete with pT classification. [3, 6]

On the other hand, traditional grading systems like that of the WHO, which are based on tumour differentation, mitotic activity and nuclear abnormalities and proven to be of major, predictive value in many other carcinomas, have failed to yield unequivocal results as disease prognosticors in ESCC. This is mainly because of the inherent reproducibility problems associated with such classification. [4, 5, 6, 7]

Indeed, this was shown to be tha case in the present study where the histological grading into three classes proved to possess a low inter-observer variations and thus was replaced grading into categories. [4, 9, 10, 11] Similary, the reproducibility of the recently proposet prognostic score, bassed on assessment of inflammatory response, pattern invasion and nuclear polymorphism, has not been extensively tested in practice as yet. [3, 4, 10, 13, 14, 15]. The failure to run the Kaplan-Meier and Ccox survival analysis precluded the testing of such a prognostic score in the present study. Due to the above reasons, the present study must rely on the lympho node status as a surrogate marker of disease progression. This information was used as the grouping variable to divide the patients into those with progressive (Node +), and less agressive (Node –) disease. [15, 16, 18]

The analysis then proceeded into the assessment of the significance of each individual variable as a predictor of 1. Lympho node status; 2. HPV status; 3. Grade of carcinoma.

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As evident from Table Nr 2, a number of these variables do seem to process a good predictive power for the previous for the lympho node status and HPV status. Noteworthy, however, is the fact that the grade of the carcinomas (for 3° and 2° grade system), was no predictive value. This is consonant with the previous data on the limited value of histological grading in the evoluation of prognosis of ESCC. [4, 10, 13, 14, 15, 16]

On the other hand, a number of interesting predictive variables were disclosed by these univariate analyses. In addition to nuclear area, G0-G1 ratio, IOD, MOD, S-phase, and HPV DNA status proved to be a significant predictor of lympho node metastases (p = 0. 006).

The variable was created by combining the results of ISH and PCR, representing the overall detection rate of HPV in these samples. It has been previously shown that DNA static cytometry is of some predictive value in ESCC and the present results are in agreement with that notion.

In histopathological analysis, results that microscopic changes suggesting HPV were no prognostic value for ESCC in a low – incidence country as Finland [6], but this notion now seems to be true with the ESCC derived from high-incidence countrise as China, as well as shown by the failure of HPV – suggestive changes to predict disease progression in either univariate or in multivariate analysis. [5, 6, 7, 9, 12, 13]

Undoubtedly, the use of HPV testing by DNA technique as a prognostic factor of ESCC sounds an attractive option. Because of the lack of predictive value for the morphological grading, HPV DNA testing might offer an applicable means to evaluate the disease outcome in these patients. [5, 6, 7, 8, 9, 10]. This approach may, however, be of limited values in countrise with a low-risk of this disease, where also the prvalence of HPV in these lesions seems to be significantly loer than in high-risk disease in China. [10, 13]

In this series, two intersting predictors of the HPV status were disclosed, MOD and nuclear roudness, which may be the surrogate markers of HPV – induced cytopathologic effects, e.g increased nuclear hyperchromasia (aneuploidy) and koilocytosis. [4, 10, 12, 19]

Better than any other variable, HPV – suggestive morphology seems to correlate in a highly significant manner with tumour grade in ESCC. Thus, changes suggesting HPV [16, 17] were almost invariably absent in poorly differentiated lesions but instead confined to highly – differentiated ones.

Notworthy, however, was the failure to find any mutual correlation between these morphological findings and HPV DNA detection (p = 0. 79). This suggest that morphological assessment of HPV – suggestive morphological changes is not a highly reproducible means to classify the ESCC lesions as HPV – positive or negative, in contrast to lesions of the genital tract. [10, 13, 14, 18, 19]

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This notion has been proposed before. Historically, morphology has been of importance as the first-line clue to HPV involvement in the lesions of ESCC. [5, 6, 12]

The univariate analysis clearly pinponted a total of 12 variables with potential to be tested as indipendent predictors of ESCC progression in multivaariate tests. When such an analysis was meticulously completed, nine of these variable remained in the model, when the limit of p < 0. 10 was used as the criteria of backward removal. Of these nine variables, statistical significance ($p < 0.05$) was reached only by 4 variables (equivalent diameter, nuclear length, nuclear perimeter and nuclear roudness). Additionally, two predictors were disclosed among the variables not shown as significant in univariate analysis, i. e adjacent dysplasia and nuclear breadth. The later was interchangeable with the nuclear length in the model, which sounds logical. The role of adjacent dysplasia is also understandably feasible; any such change around the invasive lesions is probably a sign of a "field affect" in the oesohageal mucosa, where invasive disease seems to develop through well defined precursor lesions. [4, 20, 21, 22, 23, 24]

Presence of such precursors could be regarded as a sign of a more agressive and potentially progressive of oesophageal squamous cell carsinoma. [4, 10, 16, 24]

Noteworthy, is the loss by the HPV DNA status, nuclear area, DNA index, and aneuploidy of their role as indipendent predictors of disease progression in the multivariate analysis. This is not unusual in this type of modeling, were in a variety of carcinomas, factors which look promissing in inivariate analysis loose their power in multivariate models. [4, 9, 27, 28, 29] This result, should eliminate the use of systematic HPV typing as a protential prognostic factor of ESSC. [6, 9, 12]

Our data indicated that morphologica tumour grade is of limited values as a predictor of disease progression in ESCC, which is in agreement with the experience of other authors. [13, 15, 16, 26, 27]

In our study result, than using morphological assessment which suffer from lack of reproducibility, a quantitave image analysis (static cytmetry), might have something to offer as a disease predictor in ESCC. A series of nuclear varables seem to predict disease progression in univariate analysis. Among these variables, the most significant indipendent predictors of disease progression were found in multivariate modelling as well. This notwithstanding, however, further work is urgently needed to uncover the basic pathogenetic mechanisms leading to ESCC, a major malignancy, which strictly uneven distribution on the globe and, a such, most probably also a divergent ethiology. [4, 8, 9, 10, 11, 12, 27, 28, 29, 30]

Ekucidating these mechanisms should pave the way to better prognosis of ESCC in the future.

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