ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN—POLONIA
VOL. LVI, 10 SECTIO D 2001

2nd Chair and Department of General Surgery, Medical University of Lublin
Department of General Surgery, District Specialistic Hospital, Rzeszów

ANDRZEJ DĄBROWSKI, GRZEGORZ BRAJERSKI,
GRZEGORZ WALLNER, RYSZARD ZIEMIAKOWICZ

p35 protein as a prognostic factor of squamous cell carcinoma of the oesophagus

Recent rapid advances in molecular biology raised hopes for the development of more reliable prognostic information concerning squamous cell carcinoma of the esophagus (6). In 1990, H o l l s t e i n et al. were the first to describe the mutations of the p53 gene in patients with squamous cell carcinoma of the esophagus. In the subsequent years similar observations were reported by other authors (1,14).

The mutation of the p53 gene is detected in 35%-67% of the esophageal squamous cell carcinomas (2, 3, 11). The disorders involve the loss of heterozygosity due to the point mutations. The transversions and mutations of the chain end are also frequently observed and the deletion of the 17pl3 chromosome fragment is found in 10-80% of such cases (3).

The p53 gene codes the nuclear phosphoprotein (molecular weight 50,000(53KD) bound to the large transforming antigen (T antigen) of the SV40 virus (7). In normal cells, the amount of the protein coded by p53 is small and regulatory in function. The highest values of the mRNA level for p53 and protein level are observed at the GI/S interphase of the cell cycle (5,9). The normal p53 protein is detected in the cell nucleus and shows approximately 20-30 minute half-life period (5,12). The combination of two identical molecules of the p53 protein forms a functional entity called homodimer. Its active form (phosphorylated homodimer) regulates the cell proliferation (13).

Another important finding is that DNA damage, e.g. due to ionizing radiation or chemotherapy, results in accumulation of the wild-type p53 protein (8). The increased p53 protein level favourably affects the genome stabilization arresting the cellcycle in the GI phase before synthesis and mitosis periods, thus preventing the replication of the damaged DNA and enabling its repair (13). When the damage cannot be repaired by the cell, p53 may lead the cell to apoptosis (8,10,13).

Unlike the normal p53 protein, its mutated form is capable of binding to another protein belonging to heat shock proteins (Hsc 70). The formed complex protects the p53 protein against degradation prolonging its half-life to over 12 hours (12). The p53 protein may lose its suppressive activity due to the binding (its C-terminal part) to the protein being the product of the MDMZ gene or to other proteins such as oncogenic proteins of the SV40 virus, E/b adenovirus and E6 papilloma virus (5,13,14). The product of the mutated p53 gene is incapable of forming the specific bond with DNA and initiating apoptosis (6,10,13). The mutated form is still capable of forming the bond with the normal protein form. The heterodimer produced in this way is no longer a transcription regulator, thus it cannot regulate the cell proliferation (13).

The aim of this study was to determine whether there are any relations between

the clinical signs of the tumour, depth of malignant infiltration, lymph node involvement, presence of extraregional lymph node metastasis, survival time and the p53 protein accumulation examined in biopsy specimens (before the operation).

MATERIAL AND METHODS

The analysis of the p53 protein accumulation was performed in 60 patients with squamous cell carcinoma of the thoracic esophagus who underwent the surgery in the years 1992-1995 in the 2nd Chair and Department of General Surgery, Medical University, Lublin. Among 60 patients, there were 54 (90%) males and 6 (10%) females (male : female ratio 9:1). Their age ranged from 43 to 70 years, average 57.9 ± 8.0 years. The clinical staging of carcinoma was determined using UICC classification of 1987 after careful analysis of barium swallow, endoscopy (endoscopic ultrasonography in some cases), abdominal and neck ultrasonography and CT-scans. 50 patients were heavy alcohol drinkers and cigarette smokers, 40 patients underwent chemo- or chemo-radiotherapy before the operation.

The histopathological and immunohistochemical investigations were performed in the Department of Pathomorphology, Medical University of Lublin. The endoscopic and operative specimens were fixed in 10% buffered formalin for no more than 24 h (biopsy specimens approximately 6 h). On each endoscopy 3-6 sections of the esophageal tumour were collected. The following samples were routinely collected after the operation: tumour band (3-8 full-thickness sections), esophageal normal tissue band (2 sections), gastric normal tissue band (1 section), incision lines and lymph nodes of suitable groups. The samples were embedded in paraffin, cut into 4μm sections and stained with hematoxylin, eosin (H + E) and mucicarmine. The following features were determined: depth of infiltration into the esophageal wall (pT), number of the examined lymph nodes including the nodes with metastasis and their distribution (pN), metastasis distant from extraregional lymph nodes (M_{lym+}), i.e. staging of carcinoma (pTNM).

The immunohistochemical stainings were performed on the paraffin sections from the specimens collected at endoscopy. Each time 1 representative block was selected which was cut into 4μm sections and placed on the slide covered with 3-amino-propyltriethoxysilane. The ABC Complex/HRP method was used. The sections were deparaffinized and hydrated, then heated in a microwave oven (Samsung Electronics, 750 W). The specimens in which the p53 protein expression was examined were placed for 2x5 minutes in citric buffer, pH 6.0 and slightly heated in the microwave oven. The endodogenic peroxidase was blocked by incubating the sections with 3% hydrogen peroxide for 20 minutes at room temperature. The primary antibody was incubated for 30 minutes at room temperature. The incubations with blocking sera, secondary antibodies and complex (peroxidase-labelled avidin-biotin) were carried out for 30 min at room temperature. The staining reaction was induced by incubating the sections with 3.3-diaminobenzidine chloride solution (DAB) at room temperature up to the moment when the stain was visible under the light microscope. The sections were counterstained with Mayer hematoxylin for 30 minutes and placed in Canada balsam.

The following antibodies and reagents were used: 1) monoclonal mouse antihuman p53 protein (DAKO, DO-7, code No. M 7001, (protein concentration 9.9 g/l, mouse IgG concentration 250 μg/ml); dilution 1:50; 2) biotinylated rabbit anti-mouse immunoglobulins, code No. E 354 (Dako, containing mainly IgG, concentration of specific antibodies 1.1 g/l, dilution 1:300); 3) AB Complex/HRP, code No.K 355/Dako; 4) normal rabbit serum, code No.X 902, Dako; dilution 1:20; 5) 3.3-diaminobenzidine tetrachloride (DAB), Dako.

The examination of each immunohistochemical reaction was accompanied by negative and positive

controls. In the negative control the primary antibody was replaced with the control mouse IgG 2b, No.X 0944, Dako, using the same parameters of time and temperature. The positive control consisted of the sections with adenoid colorectal carcinoma showing the intensive, positive reactions with the examined antibodies.

The accumulation of p53 protein in esophageal cell carcinoma was examined assessing the presence of immunohistochemical reaction. In randomly selected fields (areas) of the specimens, under 250 x magnification, using filters against the positive control and having determined cut-off threshold, the percentage of p53 positive cells in 1,000 neoplastic cells was calculated.

The neoplastic cells showing the reaction were assumed to be positive regardless the intensity. In each case the result was evaluated according to the 4-degree scale: negative reaction in all neoplastic cells (-), positive reaction in < 30% of the neoplastic cells (+) (focal type of the reaction), positive reaction in 30-60% of the neoplastic cells (++) (heterogenic type), positive reaction in 60-100% of the neoplastic cells (+++) (homogenic type).

METHODS OF STATISTICAL ANALYSIS

In the statistical analysis only types (++) and (+++) were regarded as the positive ones proving the excessive accumulation of the p53 protein in the cells of esophageal squamous carcinoma. The analysis concerned the relations between the above mentioned values of the marker and the following parameters: gender, localization of the neoplastic lesion grade of differentiation (post-operative histopathological examination), depth of infiltration (pT), presence of metastases to lymph nodes (pN), presence of distant (pM) metastases, carcinoma staging (pTNM), and the survival time in some cases.

A number of statistical methods were used. The differences between the means were examined by Student's t-test. Patients who died postoperatively due to complications (postoperative mortality) were excluded from survival analysis. The follow-up period ranged from 2 to 52 months. The survival curves were prepared according to the Kaplan-Meier method. The survival distributions in the examined groups were compared using the log-rank method. The effects of the studied parameters on survival were evaluated by means of the Cox proportional risk model. The effects of the parameters on the presence or absence of a given phenomenon were examined using the logistic regression model as many parameters did not show the normal distribution. The analysis involved numerous non-parametric tests, such as: Kruskal-Wallis', Sperman's non-parametric correlation, Kendal's tests. The threshold level of significance was p=0.05 (4).

RESULTS

In 7 (11.6%) cases the lesion was localized in the upper third segment of the thoracic esophagus, in 40 (66.6%) cases – in the central segment, and in 13 (21, 6) cases in its lower part. The radiological examination allowed to determine the type of neoplastic infiltration. The most frequent funnel-shaped type of the tumour was observed in 25 patients (41.7%), the spiral type was found in 21 (35%), the serrate one in 10 (16.7%) and the modular – in 4 (6.7%).

On endoscopy in 23 patients (38.3%) the infiltration was defined as the diffuse-infiltrating type, in 19 (31.7%) – ulcerating and infiltrating, in 10 (16.7%) – prominent, while in 8 (13.3%) – ulcerating but non-infiltrating.

The length of neoplastic infiltration determined on the basis of CT ranged from 30-120 mm (62 ±

2.0) while its width – 15-26 mm (average 17.3 mm). On the basis of TNM classification 19 patients (31.6%) were assumed to have stage II, and the remaining 41 (68.3%) – stage III tumour.

The morphological data of the patients are presented in Table 1. It shows that well differentiated squamous cell carcinoma was diagnosed in 8 (13.3%), moderately differentiated in 29 (48.3%), and poorly differentiated in 18 patients (30%). The absence of neoplastic cells in the operative specimens was observed in 5 cases – pTO. Those patients were previously subjected to chemotherapy and showed the complete or varied response to the treatment.

In 2 cases (3.3%) the infiltration was limited to the submucosa (pTl), in 10 (16.6%) cases involved the muscular layer (pT2), and in 25 (41.6%) cases invaded adventitia (pT3), while in the remaining 18 (30%) cases the tissues adjoining the esophagus were affected (pT4). The presence of the metastasis to the regional lymph nodes was observed in 39 patients (56.6%), in the remaining 26 patients (43.3%) the metastasis was not found. Moreover, in 11 cases (18.3%) the metastasis was present in the extraregional lymph nodes (pH_{lym+}). Complete response to initial treatment (stage 0) was found in 3 (5%) patients. The distribution of stages in the remaining group of patients was as follows: stage I in 2 (3.3%), Ha in 15 (25%), lib in 3 (5%), III in 26 (43.3%), and IV in 11 (18.3%) cases. The neoplastic cells within the vessels were detected in 33 cases (55%).

The basic clinical and pathomorphological data analyzed in patients in relation to the p53 expression before the operation are presented in Table 1. Among 60 examined specimens collected before the operation, the excessive p53 accumulation was observed in 28 cases (46.7%). The accumulation was detected only in cell nuclei. Furthermore, the focal positive reactions were observed in the cells of the basal layer of the stratified non-keritinizing epithelium of the esophagus and occasionally in the cells of the secretory part of the esophageal glands and lymphocytes.

The reaction was of the granular-diffuse character. It showed heterogeneity as to the intensity of nucleus staining and positive cell distribution in the individual cases. The nucleus colour in the positive cases ranged from dark brown (highly positive reaction) to light brown (poorly positive reaction). In some cases almost all population of the neoplastic cells showed similar intensity of nucleus colour (homogenic type). In the others the neoplastic cells with high nucleus reaction bordered on the cells with poor or negative reactions (heterogenic or focal type). In those cases the highest p53 expression was observed in the cells of the peripheral neoplastic foci or the cells from the deepest infiltrations. Furthermore, marked intensity was observed with the progression of the neoplastic cell cornification and in cells with degenerative features.

DISCUSSION

One of the assumptions of this work was that excessive p53 accumulation was a prognostic marker in the advanced esophageal carcinoma. On the basis of TNM UICC classification from 1987 the authors tried to answer the question whether the presence of such an accumulation might depend on the infiltration depth and the presence of lymph node metastasis. No component of pTNM classification showed any relation to the values of the excessive p53 accumulation.

The opinions concering the usefulness of the excessive p53 accumulation as a prognostic factor still seem to vary. S e r b i a et al. (11) in their study of 204 patients reported the results similar to the data presented in this paper. The authors failed to find any relation between the excessive p53 accumulation and the stage of carcinoma, presence of metastasis and lymph node involvement. The authors concluded that p53 is of poor prognostic value. Similar conclusions were drawn by F l e j o u et al. (1) who analyzed the p53 accumulation in 78 patients. Although the excessive accumulation was detected in 76% of their patients, it did not seem to correlate with any parameter examined. The

authors believe that the excessive accumulation alone is a bad prognostic factor. The results of the present study are in agreement with this statement.

A different opinion was presented by W a n g et al., who proved significant difference of p53 accumulation in preinvasive infiltrating (14.3%) and invasive carcinoma (68%). Moreover, they found that the intensity of overexpression increased with the depth of invasion. They also reported that the excessive p53 accumulation is related to higher invasiveness and the ability of the neoplastic cells to form metastasis. Lower percentage of the cells revealing the p53 accumulation was related to the longer survival time of the patients. Such conclusions concerned only the patients from Linxian province (China). In patients from adjacent regions the values of the excessive p53 accumulation were significantly lower.

The relations between the excessive p53 accumulation and the regions of high esophageal carcinoma risk were also noted by other Chinese authors (15). W o n g et al. (15) observed the excessive p53 accumulation in 75% of the biopsies collected from the normal esophageal mucosa. However, they could not explain the molecular basis of such a high p53 level in normal mucosa or its biological consequences. Other studies on esophageal carcinoma carried out by K a w a m u r a et al. (4) confirmed the observations of the Chinese authors. Comparing the values of the p53 accumulation in patients with superficial esophageal carcinoma and with carcinoma infiltrating the muscular membrane, the authors concluded that the excessive p53 accumulation correlated with the depth ot carcinoma invasion and was significantly higher in the infiltration spreading beyond the muscular membrane.

It should be assumed that the evaluation of the excessive protein accumulation is important in the early phase of carcinogenesis. P a r e n t i et al. (9) confirm the pertinence of such a statement. They found that the positive nuclear immunoreaction with p53 occurred in higher percentage in the invasive carcinoma compared to areas of high and low grade dysplasia. The authors came to the conclusion that in the multi-step process of carcinoma development, the p53 mutation could be an early event preceding other phenotype changes and was an indication of DNA instability. Such mutations were observed initially in the basal epithelial layer which could increase the cell ability to proliferate. This would explain the fact that the cells with "proliferative predominance"(with mutated p53) become more sensitive to genotoxic environmental damage. Cigarette smoking and drinking strong alcoholic beverages may explain harmful effects of those factors on the unstable cells with dysplasia, in which p53 has been mutated. The role of the above factors in the carcinogenesis is reported by numerous authors (14).

CONCLUSIONS

The excessive p53 accumulation in the preoperative period in patients with esophageal squamous cell carcinoma in our study does not seem to be useful for the evaluation of the staging. Since the opinions concerning this marker are controversial, it appears that further studies are necessary to define its usefulness in the clinical practice.

REFERENCES

1. F l e j o u J. F. et al: Overexpression of the p53 tumor suppressor gene product in esophageal and gastric carcinomas. Path. Res. Pract., 190, 114, 1994.

2. Hollstein M.C. et al.: Frequent mutation of the p53 gene in human esophageal cancer, Proc. Natl. Acad. Sci. USA 87, 9958, 1990.
3. Hollstein M.C. et al.: Genetic analysis of human esophageal tumors from two high incidence geographic areas: frequent p53 base substitutions and absence of ras mutations. Cancer Res., 51, 4102, 1991,
4. Kawamura T. et al.: Acceleration of esophageal squamous cell carcinoma with invasion beyond the mucosa: immunohistochemical analysis of Ki-67 and p53 antigen in relation to histopathologic findings. Cancer 77: 843, 1996.
5. Kupryjanczyk J.: Mutacja genu i akumulacja bialka p53 w rakach jajnika. Nowotwory, 46, 35, 1996.
6. Kuwano H. et al.: Expression of p53 protein in glandular differentiation admixed with squamous cell carcinoma of the esophagus. Hepato-Gastroenterology, 44, 170, 1997.
7. Lane D.P., Crawford L. V.: T antigen is bound to a host protein in SV40-transformed cell. Nature 278, 261, 1979.
8. Lowe S. W., et al.: p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell, 74, 957, 1993.
9. Parenti A. R. et al.: p53 overexpression in the multistep process of esophageal carcinogenesis. Am. J. Surg. Path., 19, 1418, 1995.
10. Prives C.: How loops, β sheets, and α helices help us to understand p53. Cell, 78, 543, 1994.
11. Sarbia M. et al.: p53 protein expression and prognosis in squamous cell carcinoma of the esophagus. Cancer 74, 2218, 1994.
12. Siedlecki J.: Nowotworowe geny supresorowe. Nowotwory, 42, 196, 1992.
13. Siedlecki J. A.: p53 – strażnik genomu. Nowotwory, 43, 84, 1993.
14. Stemmermann G. et al.: The molecular biology of esophageal and gastric cancer and their precursors:oncogenes tumor suppressor genes, and growth factors. Hum. Pathol., 25, 968, 1994.
15. Wang L. D. et al: Changes in p53 and cyclin Dl protein levels and cell proliferation in different stages of human esophageal and gastric-carida carcinogenesis. Int. J. Cancer, 59, 514, 1994.

2000.12.06

SUMMARY

Communications show that in squamous cell carcinoma of the oesophagus one can often observe overexpression of p53 protein. Wild type p53 protein suppresses cells' growth, and mutated p53 acts as onkogene.

Gene p53 mutations usually lead to stabilization and accumulation of p53 protein, and, as a result they become detectable by immunochemical methods. Overexpression of p53 has been observed in 28 (46.7%) out of 60 collected specimens. It has no connection with sex, age, histological differentiation, category (pT) of tumour, infiltration (pN) of lymph nodes and extent of metastases (pM_{lym+}). p53 protein expression has no correlation with survival predictability.

Białko p35 jako czynnik prognostyczny w raku płaskonabłonkowyn przełyku

Przedstawiona praca wykazuje, że nie ma związku między nadmierną ekspresją pochodnych p53 a rokowaniem dla pacjenta. Doniesienia wskazują na to, że w raku płaskonabłonkowym przełyku często dochodzi do nadmiernej ekspresji proteiny p53. Proteina p53 dzikiego typu tłumi wzrost komórek, zaś zmutowana proteina p53 działa jak onkogon. Mutacje genu p53 prowadzą zwykle do stabilizacji i akumulacji proteiny p53 i w rezultacie są wykrywalne metodami immunochemicznymi. Nadmierną ekspresję p53 obserwowano w 28 (46,7%) z 60 pobranych materiałów. Nie miała ona związku z wiekiem, zróżnicowaniem histologicznym, kategorią (pT) guza, nacieczeniem (pN) węzłów chłonnych i rozległością przerzutów (pM_{lym+}). Ekspresja p53 nie ma korelacji z przewidywalnością przeżycia.