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ARTICLE

P53 Overexpression as an Indicator of Overall Survival and Response to Treatment in Osteosarcomas

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The p53 gene located at chromosome 17p13 is found to be altered (allelic loss or other mutation) in multiple human cancers, including osteosarcomas. The mutated gene produces a protein with a prolonged half-life thus rendering it detectable by conventional immunohistochemistry. We examined the correlation between p53 expression and clinical prognosis as well as response to therapy. Twenty-one patients with previously untreated and histologically verified highly malignant osteosarcoma were used for this study. Biopsy material taken both prior to the start of COSS 91 protocol and at the time of surgery (ten weeks later) was examined for alterations in p53 protein expression and drug resistance. Two patients who had strong (+++) p53 protein expression and three others who became positive during the chemotherapy had significantly worse prognosis (all of them died within one year) than those who showed no p53 expression both at biopsy and after chemotherapy (all 11 patients are alive, average follow-up time: 3.5 years). All patients who showed any kind of positive p53 protein expression on initial biopsy were non-responders to chemotherapy. In contrast, 69% (9 out of 13) of those who exhibited no p53 expression on initial biopsy were responders or intermediate responders to chemotherapy. We concluded that p53 expression may be a useful prognostic factor in osteosarcomas. The direct correlation between p53 positive expression and resistance to therapy can help in identifying patients who are in need of a more vigorous or different chemotherapeutical protocol. (Pathology Oncology Research Vol 3, No 1, 15–19, 1997)

Key words: osteosarcoma, p53 overexpression

Introduction

In recent years, many genes have been discovered to play a part in the development of cancer. Mutations at chromosome 17p13 are probably the most common gene lesions in human cancers.[13,16,24] This is the location of the p53 gene which appears to act both as a tumor suppressor gene and an oncogene, depending on the circumstances. The p53 function is lost in transformed normal cells when both normal (wild type) alleles of the tumor suppressor gene are lost or inactivated.[8] The presence of one wild type copy of the gene is sufficient to interfere with oncogenesis, hence this normal allele is considered to have an antioncogenic effect.[15] However, certain mutant p53 genes induce neoplastic transformation in cells acting as oncogenes since tumor development is associated with a gain in function.[8]

The product of p53 gene is a 393-amino-acid nuclear phosphoprotein that is found in very low quantities in normal cells. Due to the short half-life of about 6 to 20 minutes of wt (wild type) p53, normal concentrations remain undetectable by conventional immunohistochemistry. However, missense mutations prolong the half-life up to six hours rendering them detectable. Therefore, the immunohistochemically detectable overexpression of p53 is currently used as an indirect indicator of p53 mutations.[3]

P53 is important in cell cycle determination, apoptosis and activation of gene expression. If a wild type gene is introduced into a transformed cell colony it will stop the

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Table 1. **P53 protein expression in stage IIA and IIB osteosarcomas**

	Biopsy material				*Surgical specimen*			
	–	+	++	+++	–	+	++	+++
IIA	4	0	0	0	3	1	0	0
IIB	11	1	3	2	9	5	1	2

cell growth at the G1 phase.[18] P53 plays a more important role in the G1-S checkpoint control of stressed cells (where p53 levels are increased) than of normal cells.[3] It facilitates DNA repair in cells exposed to DNA damaging agents, by terminating the replication and allowing time for repair. In addition, there is a clear correlation between p53 gene dosage and resistance to radiation induced apoptosis. Cells containing only one copy of the p53 gene are slightly more resistant than cells that are homozygous for p53 gene and p53 deficient mice with absence of both active copies are the most resistant. This implies that cells with one somatically mutated p53 allele may have growth advantage in the presence of DNA damaging agents over cells with two intact alleles.[17] Furthermore, p53 can act as a transcription factor.[10] Genes regulated by p53 are important in cell cycle progression, monitoring of gene amplification events and in the commitment of some cells to apoptosis.[4] Mutant p53 proteins are incapable of activating transcription from a template and in fact they block this activity of wt p53 by complexing with it.[7]

Loss of alleles at the p53 gene site (17p13) is seen in more than 75% of osteosarcomas (OS), suggesting that this alteration may contribute to the development of OS.[24] OS is the most frequent bone tumor in children and young adults, representing 8% of all childhood malignancies.[1] In regards to its etiology, genetic factors are especially important in children. For example, 40% of patients with retinoblastoma have a hereditary predisposition to other cancers. These patients with a mutation in chromosome 13q14 have a 500 times greater risk of developing OS than the normal population.[5]

Prior to effective chemotherapy the prognosis of OS patients was very poor; the 2-years overall survival ranged 5% to 20%.[20] However, with current treatment schedules the 5-year disease free survival and overall survival rates have increased to about 60 to 80%.[23] In these days the most common cause of death among OS patients is failure to respond to chemotherapy.[9] The reason is that tumors become multidrug resistant. To achieve a better therapeutical success rate it is important to select those patients with a worse prognostic outlook and those with a higher risk for multidrug resistance, in order to start a modified and more aggressive therapy regimen for them.

In this retrospective immunohistochemical study we examined the expression of p53 protein and the loss of heterozygosity of p53 gene in 21 OS patients. The aim was to evaluate the correlation between p53 expression and prognosis as well as the correlation between p53 expression and multidrug resistance.

Materials and Methods

Patients

Biopsy material was obtained from 21 patients (11 males, 10 females; age: between 15 and 24 years, with an average of 20 years) with previously untreated highly malignant OS verified by histology. The location of the primary tumor in descending order of frequency was the distal femur 43% (n = 9), proximal tibia 33% (7), proximal humerus 19% (4) and sacrum 4% (1). The occurrence rate of the different histologic variants of osteosarcoma was as follows: 12 osteoblastic (61%), 3 mixed cell (14%), 2 fibroblastic (10%), and 1-1 periosteal, teleangiectatic and small cell (5%, respectively). According to the Enneking surgical staging system[6] 17 tumors (81%) were in stage IIB and 4 (19%) in stage IIA.

Treatment

After establishing diagnosis the COSS 91 protocol was started. This consisted of 9 weeks of neoadjuvant chemotherapy, surgery on the 10th week and 14 more

Table 2. **Correlation between p53 expression and response to chemotherapy**

Case	*p53 expression*		*response to chemotherapy*	*survival after chemotherapy*
	biopsy	*surgery*		
1	+++	+++	NR	10 mo, died
2	+++	+++	NR	1 yr, died
3	++	+	NR	1,5 yr, died
4	–	–	R	3 yr
5	–	–	IR	3,2 yr
6	++	–	NR	2 yr #
7	–	+	IR	3,5 yr
8	–	–	R	4 yr
9	–	–	R	3.5 yr
10	–	+	NR	1 yr, died
11	–	–	no preop chemoth	4 yr
12	–	–	NR	2,5 yr
13	–	–	no preop chemoth	2,5 yr
14	–	–	R	2 yr
15	++	++	NR	1,5 yr, died
16	–	–	NR	5 yr
17	–	+	IR	1 yr, died
18	–	–	IR	6 yr
19	–	+	NR	1 yr, died
20	+	+	NR	7 yr
21	–	–	IR	2,5 yr

died from other reason
NR: non-responder; IR: intermedier responder; R: responder

weeks of adjuvant chemotherapy using a combination of Ifosfamid (3 g/m^2), Adriamycin (30 mg/m^2), Cisplatin (120 mg/m^2), and high-dose Methotrexate (12 g/m^2).[25]

Immunohistochemistry

The p53 antigen was detected using the streptavidin-biotin-alkaline phosphatase immunostaining method. The paraffin sections were routinely deparaffinized and pretreated, as described by Cattoretti et al.[2] by microwaving at 750 W in 10 mM citrate buffer, pH 6.0. After incubation for 20 min at room temperature with 1% BSA to block the non-specific binding of the reagents, a monoclonal mouse anti-human p53 antibody (DAKO-clone-DO-7) was applied at a dilution of 1:25 at 4 °C, overnight. This antibody reacts with both wild and mutant types of p53 protein. Slides were washed three times in Tris buffer (pH 7.6) and incubated with biotinylated anti-mouse antibody (Amersham) for 30 min at room temperature and washed twice with Tris buffer. Last, they were incubated for 45 min at room temperature with the streptavidin-biotin-alkaline phosphatase. The enzym activity was detected by incubation with its substrate (New fuchsin, DAKO). Endogenous alkaline phosphatase (AP) was inhibited by the addition of levamisol (DAKO) to substrate. Specimens were slightly counterstained with Mayers haematoxylin, and mounted with glycerol gelatin. Sections of ovarian carcinoma known to contain mutant P53 protein were used as a positive control. The same slides after omitting the primary antibody, were used as a negative control. In addition, 4 testes removed for non-neoplastic disease and 10 uninvolved normal tissues, not adjacent to tumor location, were used as additional control. All controls gave satisfactory results. Three slides of each tumor were evaluated by two of the authors without knowledge of the clinical data, and the average value was considered.

P53 scoring

Since the number of cells labelled may be more meaningful than the intensity of staining per se [11] and since the latter differed from one slide to another and sometimes, within different areas of the same slide, we were encouraged to exclude the intensity of staining from the interpretation of our results. The extent of staining was evaluated as the percentage of positively stained versus total number of tumor cells in five adjacent high power fields at a magnification of (x 400) as recently described by Lipponen et al.[19] First the entire section was screened carefully and counts were performed in representative areas, i.e. regions with the maximum fraction of positively stained cells, well fixed and free of background. Stromal components were avoided by comparing the section with the hematoxylin and eosin-stained counterpart. Tumors according to their extent of staining were classified as: (-) no obvious positive staining; (+) 5% or less of tumor cells are positive; (++) 5-50% of tumor cells are positive; (+++) more than 50% of tumor cells are positive.[12] The change in p53 protein expression was determined both in the initial biopsy specimen as well as in the surgical biopsy specimen.

In an attempt to evaluate the effectiveness of the preoperative chemotherapy and thereby multidrug resistance, patients were also divided into three groups according to the modified Salzer-Kuntschik method[22] where the percent of surviving tumor cells was determined in the surgical biopsy specimen. The three groups were: responder (living tumor cells less than 10%); intermediate responder (living tumor cells 10-50%); and non-responder (living tumor cells more than 50%). Therefore, non-responders were designated as multidrug resistant neoplasms.

Results

Alteration in p53 protein expression was observed in 29% (6/21) of initial biopsies and 43% (9/21) of surgical specimens. There were no p53 positive biopsy materials among the stage IIA tumors (*Table 1*). Among the stage IIB tumors 35% (6/17) showed positive p53 expression. In the surgical specimens only one case was p53 positive (+) among the stage IIA tumors. In contrast, 47% (8/17) of the stage IIB tumors were p53 positive. *Table 2* shows the p53 protein expression, measure of response to chemotherapy and survival time for each of the 21 cases.

Correlation between p53 expression and survival time

When biopsies showed positive p53 protein expression a shorter mean survival time was perceived (*Table 2*). Both +++ cases had metastases and local recurrences and died within 1 year. One patient with ++ in both specimens, survived only 1.5 years after having metastases and local recurrences. Two other biopsies were ++. One of them became + by the time of surgery and also survived only 1.5 years. The other turned negative by the time of surgery, but died 2 years later of a different cause. Therefore, the ++ cases had a longer survival time than the +++ cases. Only one case was + in both specimens and despite a non-responder status, the patient is still living after 7 years. Essentially, all initial biopsies that showed ++ or +++ p53 overexpression were non-responsive to chemotherapy and all the patients have died.

15 cases showed no p53 expression on initial biopsy, 4 cases became p53 positive (+) after chemotherapy. Three of these cases died within 1 year, however the remaining p53 negative patients are alive after a mean follow-up time of 3.5 years.

Table 3. **Correlation between p53 expression and response to chemotherapy**

p53 expression in biopsy material	*response to chemotheerapy (according to morphology)* *non-responder*	*intermediate responder*	*responder*
+++	2	0	0
++	3	0	0
+	1	0	0
-	4	5	4

Correlation between p53 expression and response to treatment

The sensitivity to treatment is summarized on *Table 3*. Only 19 of the 21 cases have information about the response to chemotherapy because 2 patients did not receive preoperative chemotherapy. As seen both in *Table 2* and *Table 3*, all the p53 positive cases fell into the non-responsive category. This demonstrates a positive correlation between p53 overexpression and multidrug resistance.

Among the patients who showed no p53 overexpression, 31% were responders, 38% were intermediate responders and 31% were non-responders. None of the responders turned p53 positive after receiving chemotherapy and all survived, with the mean follow up time of 3.1 years. In the intermediate responder group 40% turned positive (+) after chemotherapy and all but one patient survived.

In the non responder group 2 of the 4 patients who were initially p53 negative, became p53 positive (+) after chemotherapy, in addition, only these patients died, both within one year. Furthermore, the fact that 3 out of the 4 that turned p53 positive died within a year shows that tumors that gain p53 expression during or after neoadjuvant chemotherapy tend to show a worse prognosis than those that remain p53 negative.

Discussion

Undifferentiated OSs were shown to commonly display c-myc amplification, p53 and RB1 mutations and autocrine growth-factor production. Of these, p53 was altered in five of six OS cell lines.[14] The data available on the p53 overexpression, response to chemotherapy and length of survival in OS patients are rather contradictory.[9,21,24]

Nishikawa et al[21] analyzed p53 protein expression in 35 resected OSs. Abnormality in p53 protein expression was found in 29%. While these results showed no correlation with any clinicopathological features, the staining pattern of p53 protein seemed to correlate with aggressive growth and metastatic potential. Diffusely stained tumors had a worse prognosis than those stained focally. In addition, maximum overexpression was detected in cells in the S phase, indicating that the nuclear accumulation of p53 protein is a potentially useful prognostic factor for OS. Ueda et al[24] found p53 positivity in more than half of the tumor cells in the majority of 18 OSs. They concluded that point mutation of the p53 gene is frequently involved in the development of OS. They found however no correlation between p53 protein expression and any clinical or pathological factors.

In our study, P53 protein overexpression was detected in 29% of initial biopsies and in 43% of surgical specimens taken after nine weeks of preoperative chemotherapy. The increase in p53 expression after neoadjuvant therapy may be due to the mutagenic affects of the cytotoxic drugs used.

While there were no p53 positive biopsy materials among the stage IIA tumors, 35% of the stage IIB tumors showed positive p53 expression. In the surgical specimens only 25% of the stage IIA tumors were p53 positive compared to 47% of the stage IIB tumors. Intensive positive reactions (++ and +++) were found only in stage IIB tumors. This may imply a correlation between the tumor size and p53 expression frequency and intensity. The number of spontaneous mutations and the primary drug resistance of the tumor may increase with the size and progression of the tumor.

P53 positive (++ and +++) expression was associated with a less mean survival time than that of p53 negative cases and the ++ cases had a longer survival time than the +++ cases. However, all the patients, except one, that showed any kind of p53 expression on initial biopsy have died. In addition, 3 out of the 4 p53 negative cases which turned positive after preoperative chemotherapy also died. Lastly, the fact that neither p53 positive cases responded to preoperative chemotherapy indicates a direct correlation between p53 expression and multidrug resistance. In addition, 69% of the biopsies that demonstrated no p53 expression fell into the responder or intermediate responder category; this represents a majority. Furthermore, a gain of p53 expression during the time of neoadjuvant chemotherapy is associated with a worse prognosis. Therefore, we feel that p53 expression may be useful as a prognostic tool of OS.

References

1. *Brunat-Mentigny M and Kohler R*: Losteosarcome. Rev Prat 43: 2197-203, 1993.
2. *Cattoretti G, Becker GM, Key G, et al:* Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIBI and MIB3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. Journal of Pathology 168: 357-363, 1992.

3. *Chang F, Syrjanen S, Tervahauta A, and Syrjanen K:* Tumourigenesis associated with the p53 tumor suppressor gene. Br J Cancer 68: 653-61, 1993.
4. *Cordon-Cardo C, Latres E, Drobnjak M, et al:* Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. Cancer Res 54: 794-9, 1994.
5. *Cotran RS, et al.:* Neoplasia. *In* Cotran RS, Kumar V, Robbins SL, (eds.): Robbins Pathologic Basis of Disease. Philadelphia, W.B. Saunders Company, 1989, pp. 239-305.
6. *Enneking WF, Spanier SS, Goodman MA:* A system for the surgical staging of musculoskeletal sarcoma. Clin Orthop 153: 106, 1980.
7. *Farmer G, Bargonetti J, Zhu H, et al:* Wild-type p53 activates transcription in vitro. Nature 358: 83-86, 1992.
8. *Friedman JM and McGillivray B:* Genetic Paradigms in Human Disease. In Friedman JM, et al. (eds.): Genetics. Baltimore, Williams & Wilkins, 1992, pp. 130-4.
9. *Grundmann E, Ueda Y, Schneider-Stock R, and Roessner A:* New aspects of cell biology in osteosarcoma. Pathol Res Pract 191: 563-70, 1995.
10. *Haines DS, Landers JE, Engle LJ, and George DL:* Physical and functional interaction between wild-type p53 and mdm2 proteins. Mol Cell Biol 14:1171-8, 1994.
11. *Hall PA and Lane DP:* P53 in tumour pathology: can we trust immunohistochemistry? -Revisited!. Journal of Pathology 172:1-4, 1994.
12. *Hanna E, Bodrogi I, Institoris E, and Bak M:* A p53 expresszió és a klinikai rezisztencia összefüggésének vizsgálata hererákokban. Orvosi Hetilap 137: 59-64, 1996. (In Hungarian only)
13. *Hung J and Anderson R:* p53 functions, mutations and sarcomas. Acta Orthop Scand 273: 68-73, 1997
14. *Isfort RJ, Cody DB, Lovell G, and Doersen CJ:* Analysis of oncogenes, tumor suppressor genes, autocrine growth-factor production, and differentiation state of human osteosarcoma cell lines. Mol Carcinog 14: 170-8, 1995.
15. *Knudson AG:* Antioncogenes and human cancer. Proc Natl Acad Sci USA 90: 10914-21, 1993.
16. *Kopper L and Sebestyen A:* P53 (egy molekula, mely „végigsöpört" a daganat kutatáson). Magyar Onkologia 39: 159-164, 1995. (In Hungarian only)
17. *Lee JM, Abrahamson JLA, Kandel R, et al:* Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. Oncogene 9: 3731-3736, 1994.
18. *Levine AJ, Momand J, and Finlay CA:* The p53 tumor suppressor gene. Nature 351: 453-456, 1991.
19. *Lipponen PK:* Over-expression of p53 nuclear oncoprotein in transitional- cell bladder cancer and its prognostic value. Int J Cancer 53: 465-470, 1993.
20. *Malawer M, Link M, and Donaldson S:* Sarcomas of Bone. *In*Devita V, Hellman S, Rosenberg S (eds.): Cancer Principles and Practice of Oncology. Philadelphia, J.B. Lippincott Co., 1993, pp. 1509-1552.
21. *Nishikawa T, Yamamoto T, Mizuno K, et al:* Expression of the p53 protein in human osteosarcoma. Nippon Seikeigeka Gakkai Zasshi 68:400-406, 1994.
22. *Salzer-Kuntschik M, Delling G, Beron G, Sigmund R:* Morphological grades of regression in osteosarcoma after polychemotherapy study COSS 80. J Cancer Res Clin Oncol (Suppl) 106: 21, 1983.
23. *Serra M, Scotlandi K, Manara MC, et al:* Establishment and characterization of multi-drug resistant human osteosarcoma cell lines. Anticancer Res 13: 323-9, 1993.
24. *Ueda Y, Dockhorn-Dworniczak B, Blasius S, et al:* Analysis of mutant P53 protein in osteosarcomas and other malignant and benign lesions of bone. J Cancer Res Clin Oncol 119:172-8, 1993.
25. *Winkler K and Bielach S:* Chemotherapy of osteosarcoma. Sem Orthop 3: 48-58, 1988.