ORIGINAL ARTICLE

Expression of c-kit in human osteosarcoma and its relevance as a prognostic marker

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Aims: To examine the prognostic relevance of c-kit expression in human osteosarcomas and to evaluate the mutation status in exon 9 and exon 11 of the c-kit gene.

Methods: c-kit expression was examined in 100 human osteosarcomas by immunohistochemistry using paraffin embedded tumour tissues, and capillary sequencing of genomic DNA was performed to search for mutations in exons 9 and 11 of the c-kit gene.

Results: 20 osteosarcomas showed c-kit expression ranging from 5% to 90% (mean 5.9%; SD 16.74%). Furthermore, DNA sequences of exon 9 and exon 11 of the c-kit gene were not altered in these tumours. Overall and disease free survival analysis did not reveal any differences between patients with osteosarcoma with c-kit expression and those with c-kit negative tumours.

Conclusions: C-kit expression is not a prognostic marker in patients with osteosarcoma. The protein expression is not linked to mutations in exon 9 or exon 11 of the c-kit gene. Therefore, these exons may not function as targets for treatment modalities based on the suppression of c-kit tyrosine kinase activity.

Osteosarcoma, a frequent tumour of childhood and adolescence, represents the most common primary malignant bone tumour.¹ Despite aggressive treatment modalities such as adjuvant chemotherapy or wide tumour resection, only one quarter of patients survive for 5 years or longer. Therefore, osteosarcoma remains a major cause of fatal outcome.²

Identifying those patients who respond poorly to treatment at the time of surgery would facilitate preoperative allocation of the patient to special chemotherapy or a more aggressive regimen with the purpose of prolonging survival. Many prognostic factors have been examined such as tumour type, age at the onset of disease, location, tumour size, expression of different proteins or genetic alterations. Among clinical parameters, tumour size and response to chemotherapy seem to be of predictive value.^{3–4} The literature also refers to a whole range of immunohistochemical markers and genetic changes of different proteins,^{5–10} but their prognostic and therapeutic relevance remain doubtful.

c-kit, also known as CD117, is a protein deriving from the kit proto-oncogene after mutation and is located on the long arm of chromosome 4. The protein is the stem cell factor receptor with tyrosine kinase activity responsible for cellular processes that lead to uncontrolled growth and multiplication of cells.¹¹ Mutations of the c-kit gene induce spontaneous activity of tyrosine kinase. Therefore, tumour cells expressing c-kit protein, which has been well documented in gastrointestinal stroma tumour (GIST), have the capacity of a permanent growth stimulus.^{12–13} A therapeutic revolution for patients suffering from GIST started with the application of imatinib mesylate (Gleevec), which is a selective inhibitor of the enzymatic activity of several tyrosine kinases.^{14–15} Within the last few years, it has been determined that treatment success in GIST patients is dependent on the location of gene mutation; response to treatment with imatinib mesylate has been observed in patients with mutations in exon 9 and exon 11 of the c-kit gene.^{16–17}

c-kit expression has also been found in soft tissue sarcomas and osteosarcoma.^{18–19} This study was designed to determine whether c-kit protein is expressed in human osteosarcomas and

if the expression is of prognostic value for osteosarcoma patients.

In addition, mutation analyses of exon 9 and exon 11 were performed to find out whether protein expression was based on these genetic alterations.

METHODS

One hundred patients with osteosarcoma of bone were selected from the files of the Department of Pathology at the Medical University of Vienna. All cases were reviewed to confirm the diagnosis and a paraffin block of osteosarcoma biopsy material prior to chemotherapy and surgery was selected for immunohistochemical and PCR studies. The osteosarcoma specimens were intramedullary high-grade tumours and consisted of the following types: 54 osteoblastic osteosarcomas, 23 chondroblastic osteosarcomas, 17 anaplastic osteosarcomas, 3 teleangiectatic osteosarcomas, 2 fibroblastic osteosarcomas and 1 small-cell osteosarcoma.

The patients underwent treatment between 1985 and 2004 in the Department of Orthopaedics at the Medical University of Vienna. All patients were initially treated for primary tumours.

A specimen of gastrointestinal stroma tumour with high c-kit expression and well documented mutation in exon 9 and exon 11 was used as a control.

Immunohistochemistry

Freshly cut serial sections were used for immunohistochemical staining for c-kit and corresponding sections were stained with H&E.

Immunohistochemical staining was done on paraffin sections using a rabbit polyclonal antibody against CD117 (A4502; Dako, Glostrup, Denmark; 1:400), raised against a peptide corresponding to amino acids 963–976 at the cytoplasmic c-terminal part of c-kit. Sections were pretreated by autoclave heating in citrate buffer (pH 6.0) for 20 minutes at 1 bar. After incubation at room temperature for 1 hour, the secondary

Abbreviations: GIST, gastrointestinal stroma tumour; PDGF, platelet derived growth factor; PDGFRA, platelet derived growth factor receptor alpha

biotinylated horse anti-rabbit IgG antibody (1:300) was applied for 30 minutes, followed by incubation with the avidin-biotin peroxidase complex (Vectastain; Vector Laboratories, California, USA). The reaction was developed with diaminobenzidine as a chromogen system (Fluka-Chemie, Vienna, Austria).

Non-specific reactivity was assessed by omission of the primary antibody.

Only osteosarcomas showing equal expression intensity compared with GIST cells were scored positive. Immunoreactivity was assessed at high power magnification in 5–8 microscopic fields that showed maximum reactivity and was expressed as the percentage of positive cells per 500 tumour cells.

DNA extraction

DNA was extracted from three 10 µm thick sections of 20 paraffin embedded samples. DNA was purified using QIA amp DNA Mini Kit (no. 51306; Qiagen, Hilden, Germany), according to the tissue protocol. The samples were incubated with proteinase K (20 µl) and lysis buffer (180 µl) at 56°C for 12 hours to uncover DNA. This mixture was applied to a spin column and DNA was bound to the membrane, purified and dissolved from the membrane; 150 ng of genomic DNA was used for subsequent mutation analyses.

Mutation analysis

Gleevec-sensitive exons 9 and 11 of c-kit gene were examined for DNA mutations. The forward and reverse primers used to amplify these exons with AmpliTaq Gold (Roche, New Jersey, USA) were ATTTATTTCTCTAGAGTAAGCCAGGG and ATCATGACTGATATGGTAGACAGAGC for exon 9, and CCAGAGTGCTCTAATGACTG and CTGTTATGTGTACCCAAAAG for exon 11. After purification (MinElute PCR purification kit; QIAGEN, Hilden, Germany) gDNA products were amplified and subjected to dye terminator sequencing reaction and sequenced directly using ABI Big Dye Terminator 1.1 cycle sequence kit (Applied Biosystems, Foster City, California, USA). After another purification step using the Centri-Sep protocol (Princeton Separations, Adelphia, New Jersey, USA), cycle sequencing was performed in forward and reverse directions using an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, California, USA). The gDNA sequences of exons 9 and 11 of c-kit were analysed using GenBank sequence U63834 as the reference.

Statistical analysis

The Kruskal–Wallis test and Mann–Whitney test were used as appropriate. Overall survival was defined from the day of primary surgery until the death of the patient. Data concerning patients who survived until the end of the observation period were censored at their last follow-up visit. Death from a cause other than osteosarcoma was considered as a censoring event. Disease-free survival was defined from the end of primary therapy until first evidence of progression of disease. Univariate analyses of overall survival and disease-free survival were performed as outlined by Kaplan and Meier.

A two-tailed p-value $\leq 5\%$ was considered significant. All statistical analysis was performed using SPSS V.10.0 (SPSS, Inc., Chicago, Illinois, USA).

RESULTS

Clinicopathological data of osteosarcoma patients

The age of the 100 patients ranged from 6 to 62 years (mean 21 years); 68 were under the age of 20. None of the adult patients (age >20 years) had previous bone diseases or risk factors such as radiation therapy. The peak incidence of osteosarcomas was in the second decade of life. Sixty-five patients were men and 35 were women.

Seven tumours were located in the pelvis: 3 in the sacrum and 4 in the ilium. One tumour was located in the scapula. All other tumours were located in the extremities: 54 in the femur, 23 in the tibia, 5 in the fibula, 7 in the humerus, 1 in the intermediate cuneiform bone, 1 in the calcaneus and 1 in the first metacarpal bone. Treatment consisted of preoperative multi-agent chemotherapy according to different protocols of the Cooperative Osteosarcoma Study trials (COSS 85, $n = 3$; COSS 86c, $n = 40$; COSS 91, $n = 9$; COSS 96, $n = 47$),¹³ followed by surgical resection with wide resection margins in all cases. One patient did not receive preoperative chemotherapy.

At the time of surgery, 4 tumours were confined to bone and 96 had invaded the adjacent soft tissue. The resected specimens were analysed histologically for response to chemotherapy according to the criteria of Salzer-Kuntschik *et al.*¹⁴ This system is based on the proportion of viable tumour tissue evaluated on the cross-section of the tumour's largest diameter. Less than 10% of viable tumour tissue is considered to be a good response, whereas more than 10% of viable tumour cells is considered to be non-response; 41 cases were classified as responders and 58 as non-responders.

The duration of follow-up for survivors ranged from 1.3 months to 18.9 years (median 3.8 years) from the date of surgery. During this period 30 patients developed metastasis exclusively in the lung and three patients had a local recurrence. Twenty patients died of tumour progression. The 5-year survival rate was 65.16% and mean overall survival time was 6.4 years (SD 0.48).

Immunohistochemistry and mutation analysis

In the control material, GIST tumour cells were homogeneously positive for c-kit, showing cytoplasmic and faint membrane labelling, whereas the negative controls without application of the first antibody showed no labelling. Osteosarcomas showed positive staining in the cytoplasm and sometimes a faint membrane signal was detected (fig 1). The positivity was variable, with fields of positive tumour cells as well as areas with only single positive cells regardless of their differentiation.

Twenty tumours showed c-kit expression; labelling ranged from 5% to 90% (mean 5.9%; SD 16.74%). These specimens were further used for c-kit mutation analysis by PCR. Exon 9 and exon 11 were examined, but no genomic DNA sequence alterations were found.

Correlation with clinicopathological data; overall and disease-free survival analysis

No correlation was found between c-kit expression and patient's age (≤ 20 vs. >20 years), gender, tumour extension based on Enneking's staging system (intracompartmental lesion vs. extracompartmental lesion), location (upper extremities vs. lower extremities vs. trunk) and response to chemotherapy (responder vs. non-responder) ($p > 0.05$, Kruskal–Wallis and Mann–Whitney test).

To examine the prognostic significance of c-kit expression, osteosarcomas with c-kit expression were compared with the negative group. Univariate analysis revealed no significant difference between these groups concerning disease-free as well as overall survival ($p > 0.05$, log-rank test). Univariate analyses were also calculated for age, gender, location, extension and response to chemotherapy, revealing a significant shorter disease-free survival for patients without response to chemotherapy ($p = 0.0097$, log-rank test).

DISCUSSION

This study reveals c-kit protein expression in a wide range of highly malignant human osteosarcomas. Immunohistochemistry showed positivity in 20% of the examined specimens with variable

Table 1 Data of 20 osteosarcoma patients with c-kit mutation analyses

Patient	Gender	Age	Location	Chemotherapy	Metastases	Status	c-kit %
1	Male	10	Femur	Responder	–	Alive	25
2	Male	15	Femur	Responder	–	Alive	12
3	Male	23	Femur	Responder	–	Alive	85
4	Female	31	Sacrum	Responder	Lung	Alive	90
5	Male	18	Fibula	Responder	–	Alive	11
6	Male	21	Ulna	Non-responder	–	Alive	25
7	Male	19	Femur	Non-responder	–	Alive	10
8	Male	16	Femur	Responder	Lung	Dead	10
9	Male	14	Tibia	Responder	–	Alive	51
10	Male	16	Os pubis	No chemo	Lung	Alive	35
11	Male	25	Femur	Responder	–	Alive	10
12	Male	30	Femur	Responder	–	Alive	15
13	Male	16	Femur	Non-responder	–	Alive	15
14	Male	18	Femur	Non-responder	Lung	Alive	20
15	Male	19	Femur	Non responder	–	Alive	10
16	Male	50	Femur	Responder	–	Alive	11
17	Female	24	Scapula	Non-responder	Lung	Dead	15
18	Male	19	Humerus	Non-responder	–	Alive	80
19	Male	13	Tibia	Non-responder	Lung	Alive	50
20	Male	13	Femur	Responder	–	Alive	15

percentage of staining. Furthermore, genetic analysis of exon 9 and exon 11 was performed on the cases with immunohistochemically positive c-kit staining. Genetic alterations were not found in those cases and c-kit expression was not associated with patient's disease-free or overall survival.

A study on the involvement of c-kit in osteosarcoma oncogenesis has already been performed with a smaller but more homogeneous cohort of human osteosarcomas in children and young adults.¹⁹ c-kit expression was found in 57% of 56 examined specimens, and two thirds of immunohistochemically positive cases were associated with allelic imbalance at locus 4q12, which contains the c-kit gene. Furthermore, patients with normal status at locus 4q12 had a significantly better overall survival compared to patients with allelic imbalance, suggesting allelic imbalance of the c-kit locus to be a new prognostic factor. In a further smaller study of this working group, exons 9, 11, 13 and 17 of the c-kit gene were analysed, but mutations were not found in samples with c-kit protein expression revealed by immunohistochemistry and normal status at 4q12.²⁰

Another protein, which is involved in tumour genesis of osteosarcoma, is platelet derived growth factor receptor alpha

(PDGFRA), belonging to the same human type III family of transmembrane receptor as c-kit. We documented over-expression of PDGF-AA and PDGF- α receptor in osteosarcomas compared to benign human osteoblastomas and a strong correlation was detected between ligand and receptor expression in osteosarcomas.²¹ High PDGF-AA expression was a predictor of worse prognosis.²² Although molecular studies and PDGFRA mutation status in these tumours have not been performed, PDGFRA might also be a therapeutic target for osteosarcoma patients as documented in an in vivo animal model.²³

Another interesting approach is the study by Mintz *et al*; they found that a genetic expression signature of osteosarcomas identified tumours with a poor response to preoperative chemotherapy, and identified potential target candidates to customised induction therapy.¹⁰ The gene expression profiles of biopsy samples obtained from responders and non-responders were compared to identify the genes predictive of poor survival. The results of this study showed that resistant tumours had an increased ability to express osteoclastogenesis, tumour progression, and extracellular matrix remodelling genes.

In conclusion, we have shown that c-kit is expressed in some human osteosarcomas but protein expression is not of predictive value for the outcome of osteosarcoma patients. Mutations in exon 9 and exon 11 of the c-kit gene were not detected in the group showing protein expression. Therefore, other genetic changes might play a crucial role in tumour genesis, and treatment modalities based on the suppression of c-kit tyrosine kinase activity needs further basic genetic research in human osteosarcoma.

Figure 1 A highly malignant osteosarcoma showing positive membranous and cytoplasmic labelling in a large number of tumour cells (magnification $\times 400$).

Take-home messages

- Although c-kit has been frequently cited as prognostic marker in some human malignancies, c-kit does not predict the outcome of patients with osteosarcoma.
- The expression of c-kit in osteosarcoma cells is not based on genetic changes in exon 9 and exon 11 of the c-kit gene.

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The procedures followed in this study were in accordance with the guidelines of the human ethics committee of the Medical University of Vienna.

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