Epidermal growth factor receptor (EGFR) status in chordoma

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Chordoma is a rare tumour arising from the embryonal remnants of a notochord occurring most commonly in the sacrococcygeal as well as head and neck locations. Current treatment includes surgery and/or proton beam radiotherapy. In several cases especially in the head and neck location, surgery is not advised. Proton beam therapy is not always effective enough to eradicate the tumour. Additional modes of therapy are needed. One of the current therapeutic approaches in various tumours is targeted therapy and one of the targets is EGFR. The aim of this study was to evaluate EGFR expression and *EGFR* gene status of chordoma. Twenty-one cases of chordoma were retrieved from the in-house and consultation files of the Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology in Warsaw. Immunohistochemistry with an anti-EGFR antibody and FISH was performed on slides obtained from representative archival paraffin blocks. In our study 81% of cases of chordoma showed low to high EGFR expression in immunohistochemistry. In six cases (26.6%) the FISH results for *EGFR* were classified as positive (an average *EGFR* copy number ≥ 4 per cell). There was one case of chromosome 7 aneuploidy reported.

Key words: chordoma, epidermal growth factor, fluorescence in situ hybrydization, immunohistochemistry

Introduction

Chordomas are rare, slowly growing, locally aggressive and metastasizing primary neoplasms of bone with an incidence rate of < 0.1/100,000/year and comprise 1-4% of all primary bone tumours [1]. They arise from embryonic remnants of the notochord and show epithelial as well as mesenchymal differentiation. Chordomas are located mostly in the sacrum (50-60%), skull-base region (25-35%), cervical vertebrae (10%) and thoracolumbar vertebrae (5%). They occur predominantly in the 5th and the 6th decade with median age of 60 years; nevertheless, skull base tumours have been reported in children and adolescents. Clinical presentation depends on location of the malignancy. The major symptoms are pain and a broad spectrum of neurological deficits. Chordomas are ranked among neoplasms with low malignant potential although in approximately 43% of cases distant metastases to lung, bone, soft tissue, lymph node, liver and skin are reported. The mean overall survival time was assessed as 6 years with survival rate 70% at 5 years and 40% at 10 years [1, 2]. According to the current investigations adverse prognostic factors in chordoma include larger tumour size, performance of an invasive diagnostic procedure outside the major tumour centre, inadequate surgical margins, microscopic tumour necrosis, Ki-67 index above 5% and local recurrence [1-5].

The treatment of chordoma comprises surgery and radiotherapy. Surgical outcomes depend mainly on the location of the tumour and the initial size [6, 7]. Patients receive maximal benefit if an *en bloc* resection can be performed with margin-free pre-

servation. It generally can be effective in chordomas occurring in the thoracic and lumbar spine and sometimes even in selected cases of cervical spine tumours [8]. Radiotherapy has been demonstrated to be a valuable modality for local control and has an influence on the total overall survival [9]. Curative treatment of "clinically malignant" intracranial chordomas is extremely difficult for three major reasons: radical surgery to the base of the skull is almost impossible without permanent damage; relatively high radiation doses are needed for local control; and proximity to the medulla oblongata and brainstem, which have a lower radiation tolerance, limits the possibility of conventional radiotherapy in higher doses [10, 11]. Proton beam therapy in a clearly defined area makes it possible to increase the total radiation dose compared with the other external radiotherapy modes and currently is used in the treatment of skull base tumours [12, 13]. Chemotherapy is poorly active and remains an option in tumour recurrence [14, 15].

Recent interest has focused on molecular targeted therapeutic strategies. In the initial study performed by Casali *et al*. [16] six patients were treated with imatinib mesylate, which acts by blocking c-Kit and platelet-derived growth factor receptor β (PDGFR-β). Subsequently in several investigations PDGFR and its phosphorylated forms were expressed in chordomas [17-20]. Another therapeutic target is epidermal growth factor receptor (EGFR). There are scarce studies reporting expression of EGFR as well as c-Met [21] and a single case of administration of anti-EGFR drugs to the patient [22]. The aim of our study was to evaluate EGFR protein expression and *EGFR* gene status in chordoma.

Materials and methods

Twenty-one cases of chordoma were retrieved from the in-house and consultation files of the Maria Skłodowska-Curie Memorial Cancer Centre Institute of Oncology in Warsaw. The cases were diagnosed between 2000 and 2008. The mean age was 57.1 years (25-78 years). There were 13 (61.9%) men and 8 (38.1%) women. The mean age of men and of women was similar, respectively 52.4 vs. 64.9 years. The location included sacrococcygeal region in 11 (52.4%) cases and skull base in 10 cases (47.6%). There were no significant differences of age between these two locations. Among observed groups of patients the outcome was as follows: stabilization of illness in 10 cases (47.6%), progression in 7 cases (33.3%), regression in 1 (4.8%) and no data were available in 3 cases (14.3%). The mean time of follow-up in the subgroup of patients with stabilization was 18.6 months (range 3-54 months). The patient with regression remained under observation for 39 months. Progression was defined as recurrence (in 3 cases) or distant metastases (in 4 cases).

Immunohistochemical analysis

Immunohistochemistry with an anti-EGFR antibody (DAKO, clone 2-18C9) was performed on slides obtained from the representative archival paraffin blocks of all 21 cases. Deparaffinized sections were enzymatically pretreated with proteinase K for 5 minutes at room temperature. Sections were blocked for peroxidases in 0.3% H_2O_2 solution for 5 minutes and in methanol for 30 minutes, and incubated with primary anti-EGFR antibody for 30 minutes at room temperature in a humidity chamber. For detection the EnVision Plus system (DAKO, PharmDx EGFR) was used. Immunohistochemical staining for EGFR was evaluated following the criteria recommended by the manufacturer: 0, no discernible staining or background type staining; 1+, equivocal discontinuous membrane staining; 2+, unequivocal membrane staining with moderate intensity; and 3+, strong and complete plasma membrane staining. More than 5% of cells were required to meet the criteria for EGFR analysis. Additionally S100 (DAKO, 1 : 100), EMA (DAKO, 1 : 50) and cytokeratins CKAE1/AE3 (DAKO, 1 : 100) stainings were done according to routine procedures.

FISH analysis

All procedures were performed according to the manufacturer's protocol (Abbott-Vysis). Sections were deparaffinized in xylene (2 × 10 minutes, room temperature), dehydrated in 99.98% ethanol (2 × 5 minutes, room temperature) and air-dried. Tissue sections were then treated with 0.2 M HCl (20 minutes, room temperature), rinsed with distilled water (3 minutes, room temperature) then in standard saline citrate (2 × SCC, 3 minutes, room temperature) and immersed in pre-treatment solution (30 minutes, 80°C, water bath). The pre-treatment step was followed by rinsing samples in distilled water (1 minute, room temperature) and in standard saline citrate (2 × SSC, pH = 7,2, 2 × 5 minutes, room temperature). Slides were transferred to the protease working solution (Abbott-Vysis) (25-45 minutes, 37°C, water bath). Tissue sections were then rinsed in 2 × SSC (2 × 5 minutes, room temperature), dehydrated in graded series of alcohol (70, 80, 96%, 1 minute each, room temperature) and air-dried. Dual-colour probe cocktail including EGFR (Spectrum Orange) and chromosome 7 (CEP7, Spectrum Green) probe was applied (Abbott-Vysis). The quantity of probe mix applied was dependent on the size of the slide and varied from 10 to 30 μl per slide. In order to prevent vaporization the slides were covered with a cover slip and sealed with rubber

cement. Specimen and probe DNA were denatured by placing the samples in Thermo-Brite (1 minute, 85°C). Hybridization was carried out under the cover slip (overnight, 37°C). Unbound probe was washed away with Post-Hybridization Solution 1 (Abbott-Vysis, 0.4 × SSC/0.3% NP-40, 2 minutes, 72°C, water bath) followed by washing in Post-Hybridization Solution 2 (2 × SSC/0.1% NP-40, 30 seconds, room temperature). Tissue sections were then air-dried in darkness and counterstained with DAPI (Abbott-Vysis). Slides were evaluated for *EGFR* gene status using Olympus BX40 microscope equipped with filters: Spectrum Orange, FITC, DAPI monofilters and triple-band pass (rhodamine/FITC/DAPI) filter. Samples were scanned at 100 × magnification, and *EGFR* and CEP7 signals were counted at 1000 × magnification in at least 60 cells. The average number of *EGFR* gene copies per cell was evaluated. The case was considered *EGFR*-positive if the average *EGFR* copy number was higher than 4 or clusters of *EGFR* gene were observed in more than 30% of examined cells or if the ratio *EGFR*/*CEP7* was ≥ 2.

Results

Most of the cases (95.2%) were strongly S100, EMA and CKAE1/AE3 positive. These reactions supported the histopathological diagnosis of chordoma.

Seventeen cases (81%) showed low to strong expression of EGFR, and four cases were negative (19%). The pattern of expression in all specimens was mixed, membrane and cytoplasmic with stronger membrane staining. A summary of the EGFR immunostaining evaluation including intensity of staining and percentage of stained cells is presented in Fig. 1 (A, B).

Six cases were classified as FISH positive (26.6%) with an average *EGFR* copy number higher than 4. There were no cases with *EGFR* amplification – in the whole investigated group *EGFR*/*CEP7* ratio was below 2. Aneuploidy of chromosome 7 was reported in a single case. In two cases two cell populations were seen – the first containing small, round cells without EGFR gene alterations and the second with large, ovoid cells demonstrating increased *EGFR* copy number. These cases were counted as FISH positive. In FISH positive chordomas 3 patients had illness stabilization, 2 progression and in one case there were no data available. A summary of FISH results including clinical outcome is presented in Table 1.

Examples of HE, EGFR immunostaining, a FISH positive result and additional immunoreactions (CKAE1/AE3) in chordoma are shown in Fig. 2.

Discussion

Chordomas are poorly sensitive to chemotherapy and currently there are no effective drugs which could support surgical and radiotherapeutic protocols of treatment [15]. Recent studies have concentrated on molecular-targeted agents. The up-to-date reports have confirmed that imatinib mesylate, acting as an anti-PDGFR drug, is active in chordomas, providing a partial response in a significant proportion of patients. It may improve symptoms and the progression-free interval [16-19].

The other cell-signalling pathway which seems to be associated with development of chordoma involves the molecules focused on receptor tyrosine kinases, i.e. EGFR. One of the earliest recognized properties of cancer cells is their growth factor self-sufficiency, which is achieved by activation of cellular proto-oncogenes, either by mutation or overexpression [23]. Further evidence of the importance of growth factors and their receptors in tumour progression includes the association between malignancy and aberrant signal transduction mediated by growth factor receptors, such as members of the human

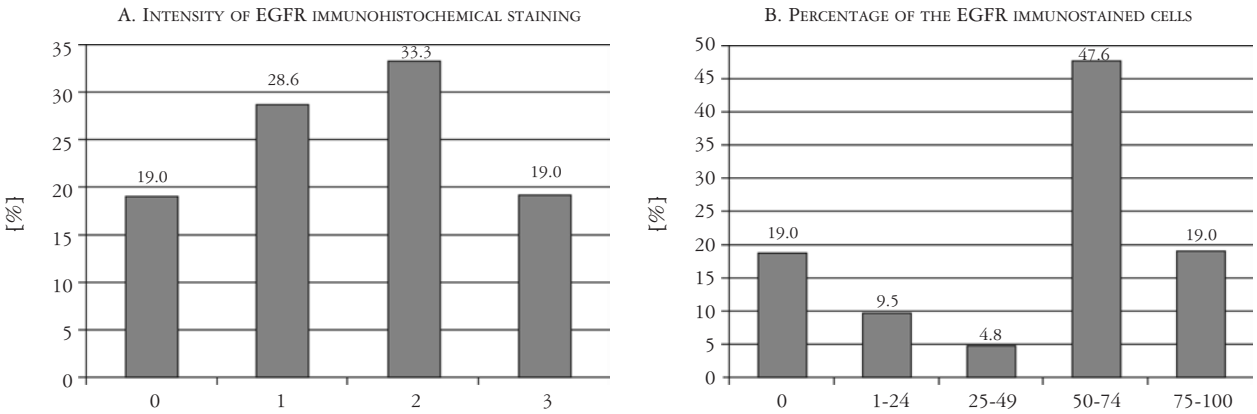

Fig. 1. Summary of immunohistochemical evaluation of EGFR (A – intensity of staining, B – percentage of stained cells)

Table I. Evaluation of *EGFR* gene in FISH (FISH positive results are bold)

N	PATIENT	RATIO	*EGFR*	*CEP7*	OUTCOME
1	SW	1.08	2.03	1.88	LD
2	MM	ND	2.10	ND	LD
3	**NN**	**1.75**	**4.60**	**2.63**	LD
4	JG	0.98	3.07	3.13	P
5	**PK**	**1.21**	**8.55**	**7.05**	P
6	**MH**	**0.92/1.07**	**2.45/8.44**	**2.65/7.88**	P
7	MB	0.98	1.92	1.96	P
8	WG	1.06	3.23	3.05	P
9	SN	1.14	2.93	2.57	P
10	WN	ND	1.45	ND	P
11	WJ	1.12	2.48	2.22	R
12	BL	0.95	2.15	2.27	S
13	LS	1.01	2.42	2.40	S
14	RC	1.09	2.63	2.40	S
15	**RD**	**1.09**	**6.05**	**5.50**	S
16	**JD**	**1.64/0.82**	**28.01/4.08**	**17.06/4.9**	S
17	ZP	0.93	1.92	2.05	S
18	TK	1.01	2.38	2.35	S
19	WW	0.95	2.47	2.58	S
20	**JK**	**1.94**	**4.70**	**2.42**	S
21	ZK	1.16	2.67	2.30	S

ND – technically non-diagnostic material
LD – lack of data
P – progression
R – regression
S – stabilization

EGFR (ERBE) family [24, 25]. In fact, an activation of ERBE family members leads not only to increased cell proliferation, but also to cancer cell resistance to growth-inhibitory cytokines and expression of selective immunosuppressive and pro-angiogenic cytokines and chemokines, thereby creating an environment that favours tumour progression [26, 27]. Therapeutic reagents targeting ERBE family members, in particular EGFR, have proven successful for the treatment of breast, colon, lung, and pancreatic cancers [27-29]. Thus, not all tumours expected to respond to these therapeutics are indeed sensitive to them and a proportion of the cases develop drug resistance to EGFR inhibitors [30].

The first report concerning expression of EGFR, c-neu oncogene product and transferrin in chordomas originates from Japan and was published in 1990 [31]. The authors examined 13 cases of chordoma, with and without metastasis. Immunohistochemically 61% of cases were positive for EGFR and 85% for c-neu oncogene product, whereas all cases were negative for transferrin receptor. The conclusion from that report was a suggestion of possible link between the expression of cellular genes associated with the growth of neuroectodermal cells and the growth of chordoma known to arise from embryonal rest of the notochord. After 25 years Weinberger *et al*. [21] assembled a cohort of 12 chordomas and examined the expression of hepatocyte growth factor/scatter factor receptor (c-Met), HER2/neu and EGFR compared with 17 other malignancies. The immunohistochemical analysis revealed predominant strong expression of EGFR (strong 50%, moderate 33%, weak 17% EGFR expression), and c-Met (strong 58%, moderate 17%, weak 8% c-Met expression). The HER2/neu expression displayed a spectrum of intensities ranging from no expression (25%) to very strong expression (33%). The authors infer that chordomas, like many other solid tumours, express HER2/neu, EGFR and c-Met and present strong expression of both EGFR and c-Met. They suggest that EGFR inhibitors represent a potentially valuable experimental treatment option for refractory chordoma but further studies are required. In 2008 Fasig *et al*. [22] published results of the immunohistochemical analysis of receptor tyrosine kinase signal trans-

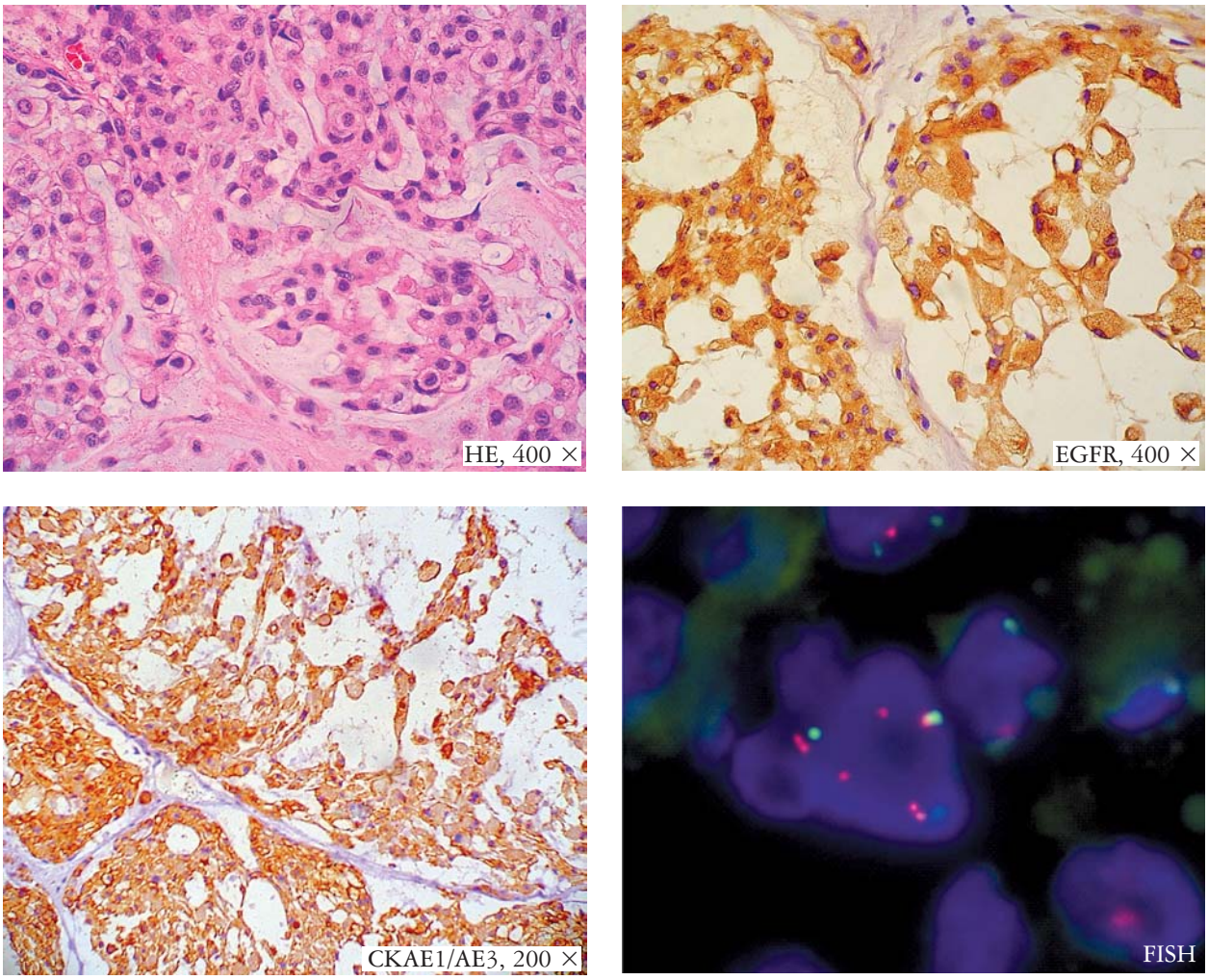

Fig. 2. Chordoma – HE, EGFR, CKAE1/AE3 immunostaining and FISH positive result

duction activity in 21 cases of chordoma. PDGFR-β, EGFR, KIT and HER2 were detected in 100%, 67%, 33% and 0% of cases respectively. The phosphorylated isoforms p44/42 mitogen-activated protein kinase, Akt, STAT3, indicative of tyrosine kinase activity, were detected in 86%, 76% and 67% of cases respectively. Weak staining of phosphorylated EGFR was evaluated in 43% of chordomas. The authors did not observe statistically significant correlations between the expression of any of the markers studied and disease-free survival or tumour location. In our study immunohistochemically 81% of cases were evaluated as positive and 19% as negative. The discrepancies between our results and those of Weinberger *et al*. [21] may originate from dissimilar immunohistochemical scoring method. In our study the EGFR staining in order to be considered a positive result had to exceed the cut-off point which was 5% of positively stained cells. According to the latest reports concerning non-small cell lung carcinoma and colorectal carcinoma the cut-off point is an important topic and still remains a subject of controversy [30, 33]. In recent recommendations a minimum of 10% of lung carcinoma and 1% of colorectal carcinoma cells must be positive to be regarded as positive [34, 35]. The "golden rule" of one positively stained neoplastic cell seems to not be obligatory any more. In addition, from the clinical point of view, most studies have failed to show any relationship between EGFR expression determined immunohistochemically and the clinical activity of anti-EGFR drugs, e.g. cetuximab, which has also been shown to have clinical activity in patients with colorectal cancer that is negative for EGFR [36]. Similarly, in a prospective clinical trial, the response to treatment with panitumumab in patients with metastatic colorectal carcinoma was similar whether EGFR protein expression was high, low, or negative, as assessed by immunohistochemical methods [37]. These data suggest that immunohistochemical testing for EGFR is not an optimal method for

identifying patients who may respond to treatment with anti-EGFR drugs. Studies on finding a marker which would facilitate distinguishing patients who may obtain maximal benefit from anti-EGFR therapy are continually ongoing [30]. One of the markers is thought to be an increased *EGFR* copy number with balanced polysomy detected by FISH [38-40]. In a high proportion of cancer cells, approximately 25 to 40% of patients with non-small-cell lung carcinoma, squamous-cell carcinoma of the head and neck, or colorectal carcinoma, quantitative and qualitative changes of the *EGFR* gene are present [41, 42]. Moreover, the predictive role of increased *EGFR* copy number has also been evaluated in patients with metastatic colorectal carcinoma in a series of retrospective studies [43]. In our study 6 (26.6%) out of 21 cases of chordoma were FISH positive with EGFR signals of more than four. There was one case with the aneuploidy of chromosome 7. There were no significant differences between clinical data (age, gender, outcome) or immunohistochemical staining and FISH result. We did not come across reports concerning gene status of *EGFR* in chordoma by searching the PUBMED database.

To date, the only study characterizing the effectiveness of cetuximab/gefitinib in the therapy of sacral chordoma is a single case report published in 2006 [22]. A patient with a sacral chordoma and pulmonary metastases received an initial surgery and radiotherapy for a local recurrence. After 11 months another local recurrence and progression of the pulmonary metastases were documented. As tumour biopsies revealed expression of EGFR immunohistochemically, an individual approach with a combination of cetuximab and gefitinib was followed. During the therapy the local recurrence and pulmonary metastases showed a partial response over the follow-up period of 9 months. After that case report no further clinical trials on the administration of EGFR inhibitors in chordoma were conducted.

In conclusion, we confirm that the majority of chordomas express EGFR immunohistochemically. Nevertheless, it translates only into *EGFR* gene alterations only in about one-fourth of the cases. Because of the rarity of chordomas only a few oncology centres have developed extensive experience in its management. The largest clinical reports have been based on series of cases collected over a long periods of time and seldom treated according to the same proceedings. To understand the molecular changes and signalling pathways involved in the development of chordoma, further studies are needed.

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