HER2, EGFR and TOPIIA gene amplification and protein expression in synovial sarcoma before and after combined treatment

Konrad Ptaszyński[1], Anna Szumera-Ciećkiewicz[1], Klara Zakrzewska[1], Tomasz Tuziak[1], Anna Mrozkowiak[1], Piotr Rutkowski[2]

[1]Department of Pathology, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Warsaw
[2]Department of Soft Tissue/Bone Sarcoma and Melanoma, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Warsaw

Synovial sarcoma (SyS) occurs mostly in young adults and is characterized by an aggressive course. Combined treatment including chemotherapy, radiotherapy and surgical excision of the tumour is still not satisfactory, with mean 5-year survival of 30-50%. New targeted treatment options have appeared recently, e.g. HER2 and EGFR antagonists. Initial studies have revealed immunohistochemical overexpression of the EGFR in SyS; therefore trials with EGFR antagonist therapy have commenced. The aim of our study was to evaluate the status of HER2, EGFR and TOPIIA in SyS before and after combined therapy. Immunohistochemistry and FISH tests were performed. Significant discrepancies between protein expression and gene status were found. The authors discuss the potential reasons for that phenomenon.

Introduction

Synovial sarcoma (SyS) comprises 10-15% of all sarcomas of the soft tissues. It mainly occurs among young adults and predominantly is situated near the joints of the upper and lower extremities. It is ranked among high grade sarcomas with aggressive course and a high rate of local recurrences and distant metastases, mostly to the lungs (50% and 40% respectively) [1, 2]. The treatment consists of combined therapy including neoadjuvant chemotherapy, preoperative radiotherapy and surgical excision of the tumour. Mean 5-year survival of patients who undergo combined treatment is approximately 30-50% [3, 4].

On the basis of the histological structure, three subtypes of SyS are identified: monophasic with spindle cell sarcomatous component, biphasic composed of epithelial and sarcomatous components, and poorly differentiated [4]. The distinctive genetic feature of nearly all SySs is a translocation t(X;18) which leads to expression of the fusion protein SYT-SSX [4, 5].

Recently, new therapeutic possibilities for various solid tumours have appeared owing to the selective treatment, e.g. tyrosine kinase inhibitors of the growth factor receptors from family ErbB: EGFR (ErbB1) and HER2 (ErbB2). Neoplasms demonstrating expression of the growth factor receptors are treated with anti-EGFR monoclonal antibodies (e.g.: cetuximab, panitumumab) and small-molecule EGFR tyrosine kinase inhibitors (e.g.: erlotinib, gefitinib). The mechanisms of action of EGFR inhibitors include blocking cancer cell proliferation, promoting apoptosis, inhibiting invasion, metastasis and tumour-induced neovascularisation [6, 7].

Overexpression of HER2 was confirmed in a number of malignancies including breast, ovarian and pancreatic cancer as well as osteogenic sarcoma. The clinical course of breast cancer with overexpression (IHC) and amplification (FISH) of HER2 is associated with worse prognosis, more

advanced stage and a higher risk of metastases. In HER2 positive breast cancer, according to the current recommendations, treatment including trastuzumab is recommended [8]. It has a significant influence on the course and prognosis. There are few investigations, mostly carried out on small groups of patients, trying to determine the status of HER2 in SyS. The results reveal moderate immunohistochemical overexpression of HER2 protein and increased *HER2* gene copy number with balanced polysomy in 10-40% of SyS cases [9, 10]. The role of HER2 in the development of SyS has not been finally defined.

Current investigations have also shown increased expression of the epidermal growth factor receptor (EGFR) in SyS. An attempt to start treatment of SyS with EGFR antagonists was initiated [3, 11]. The experience gathered in using this group of therapeutics in lung cancer indicate that efficacy of EGFR antagonists depends on somatic EGFR gene mutations or increased EGFR copy number. Several studies have confirmed that quantitative and qualitative genetic changes have an influence on the clinical response to treatment with erlotinib and gefitinib [12-14]. Current knowledge on the mechanisms of EGFR overexpression in soft tissue sarcomas is limited.

An alternative treatment of SyS is based on anthracyclines, but this chemotherapy is highly toxic [15]. Markers which would facilitate selecting groups of anthracycline sensitive responders are being sought. Studies on evaluating expression and amplification of gene topoisomerase IIA (TOPIIA), which is an enzyme blocked by anthracyclines, in breast cancer unambiguously showed that patients with increased TOPIIA copy number, confirmed by FISH method, receive the most benefits of adding anthracyclines to the standard chemotherapy [15, 16]. According to the literature, in SyS there are only a few studies on the expression and amplification of TOPIIA [17].

Material and methods

The material was obtained from patients diagnosed and treated at the M.C.S. Memorial Cancer Centre and Institute of Oncology in Warsaw between 2000 and 2006. Ten patients with the initial diagnosis of SyS were qualified for the investigation (6 women and 4 men). The average age of the patients was 34.3 years (17 to 54 years old). Material from the primary tumour was obtained by open biopsy in all the patients. After confirmation of SyS patients were qualified for the combined therapy according to Eilber's scheme [18]. Two courses of neoadjuvant chemotherapy and fractional radiotherapy with a total dose of 2000 Gy were applied and then surgical tumour extirpation with preservation of an adequate tissue margin was performed. Each patient received 6 courses of post-operative chemotherapy (ifosfamide). During the treatment the patients were monitored; local recurrence and distant metastases were reported. Among three patients distant metastases to the lungs were identified and after second line chemotherapy surgical excision was performed. Two patients were disqualified for operative treatment because of multiple metastases in both lungs. One of the patients after local recurrence had an expanded surgical intervention and the extremity was amputated.

Immunohistochemistry

All specimens were fixed in 10% buffered formalin and embedded in paraffin according to standard procedures. Serial sections (4 μm thickness) were used for haematoxylin and eosin staining, immunohistochemistry, and FISH analysis. Immunohistochemical study was performed using DAKO (Denmark) antibodies against CK7 (clone OU-TL 12/30, dilution 1 : 50), CK19 (RCK 108, 1 : 50), HER2 (polyclonal), EGFR (2-18c9, 1 : 100) TOPIIA (Ki-S1, 1 : 50). Paraffin-embedded sections of tumour were deparaffinized, dehydrated and heat-treated for antigen retrieval for CK7 and CK19 in a water bath at 96°C TRIS/EDTA buffer, pH 9.0 for 30 min, HER2 in water bath at 96°C 10 mM citrate buffer pH 6.0 for 40 min, EGFR by proteinase K for 5 min at room temperature, TOPIIA in 10 mM citrate buffer pH 6.0 for 20 min in a 600-watt microwave oven and then cooled to room temperature. Subsequently all sections were blocked in 0.3% H_2O_2 in methanol for 30 min, and incubated with primary antibody for 30 min at room temperature in a humidity chamber. For detection, a DAKO Real ENVISION System HRP (CK7, CK19, TOPIIA), HerceptTest (HER2) and EGFR PharmDx (EGFR) were used. Immunohistochemical staining for EGFR and HER2 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 10% of the cells were required to meet the criteria for HER2 and EGFR analysis. Scores of 2+ and 3+ staining levels were considered to be EGFR overexpression, scores of 3+ staining levels were considered to be HER2 overexpression. Presence of TOPIIA-positive cells was determined by assessing the proportion of positive tumour cell

nuclei within a neoplasm; the percentage of positively stained nuclei was calculated and was expressed according to the scale as follows: 0 discernible staining or background type staining, 1+ 1-10%, 2+ 10-33%, 3+ 33-66%, 4+ 66-100% stained nuclei. The immunostainings for CK7 and CK19 were classified as negative <1%, weak 1-10%, moderate 10-50%, strong when > 50% membrane cell stained.

FISH analysis

FISH analysis for SYT, EGFR, HER2 and TOPIIA was carried out using the SpectrumOrange (SYT, EGFR, HER2, TOPIIA)/ SpectrumGreen (CEP7, CEP17, CEP18) probe (Vysis-Abbott Laboratories) according to the manufacturer's protocol. Sections were incubated at 56°C overnight, deparaffinized, and dehydrated in 100% ethanol. After incubation in 2 × saline sodium citrate buffer (2 × SSC, pH 7.0) at 80°C for 25-30 min, sections were digested with proteinase K (0.25 mg/ml in 2 × SSC; pH 7.0) at 38°C for 17 min, rinsed in 2 × SSC at room temperature for 5 min, and dehydrated in a series of increasing concentrations of ethanol (70%, 80%, and 100%). The SYT/CEP18, EGFR/CEP7, HER2/CEP17, TOPIIA/CEP17 probe sets were applied to the selected areas based on the presence of tumour foci on each slide, and the hybridization area was coverslipped and sealed with rubber cement. The slides were incubated for co-denaturation of the chromosomal and probe DNA: SYT at 73°C for 5 min, EGFR at 85°C for 1 minute, HER2 at 72°C for 2 min and TOPIIA at 75°C for 1 minute and subsequently were hybridized at 37°C for 18-24 hours. Post-hybridization washes were performed in 0.4 × SSC/0.3% NP-40 at 73°C for 2 min in a water bath and 2 × SSC/0.1% NP-40 for 2 min at room temperature, air-dried in darkness, counterstained with 4',6'-diamidino-2-phenylindole (DAPI), and a coverslip was applied. FISH analyses were performed independently by two authors who were blinded to the clinical characteristics of the patients and to all other molecular variables. For EGFR FISH analyses, 80-100 nuclei were scored for signals from both DNA probes using an Olympus microscope equipped with a triple-pass filter (DAPI/Green/Orange; Vysis) at a magnification of 1000 ×. The cases were considered to be amplified when the average copy number ratio was ≥ 1.5. For each FISH preparation, known positive and negative cells were used as controls.

The statistical analysis was made on the standard equipped PC program Statistica 7.

Results

The general characteristics of patients are depicted in Table I.

On average patients remained under observation at the hospital for 38.6 months (13-79 months). The mean time free from recurrence and metastasis was 33.7 months (1-79 months). Three patients (30%) approached generalization of the illness and distant metastases in the lungs appeared after mean time of 13.6 months (1-28 months). One patient had a local recurrence. The mean largest diameter of the primary tumour was 9.58 cm (4-20 cm) and the percentage of necrosis after treatment was 23.5% (0-75%). Among primary tumours 5 were classified as monophasic, spindle cell and 5 as biphasic SyS. After the treatment two cases classified initially as biphasic had the appearance of monophasic, spindle cell in the subsequent biopsies and postoperatively (monophasic vs. biphasic: 7 vs. 3). Metastatic tumour showed the appearance of monophasic, spindle cell and poorly differentiated SyS. A recurrent disease showed histology of poorly differentiated SyS. Altogether 23 SyS specimens, originating from 10 patients, were tested.

Immunohistochemistry

In all cases of the biphasic subtype the epithelial part of the SyS showed a strong positive membranous reaction for CK 7 and CK 19. Three specimens (13.04%) were weakly HER2 positive. EGFR staining was strongly positive in 21 specimens (91.3%). The summary of the EGFR evaluation is presented in Fig. 1. In five specimens the nuclear reaction for TOPIIA was negative (21.74%) and the remaining ones (78.26%) were positive with mean percentage 33-66% (2+).

FISH

In all cases the translocation t(X;18) was confirmed by a FISH detection of *SYT* gene split. No evidence of an amplification of *HER2* or *EGFR* gene was found. In one case the ratio for *TOPIIA* was 1.5 and it may be classified as weakly positive. The percentage of technically inadequate material was 8.7%. A summary of the FISH evaluation is displayed in Table II.

Discussion

The hallmark of SyS is the balanced translocation between chromosome X and 18 t(X;18) (p11,2; q 11,2) (5). It is estimated that the percentage of SyS with the translocation depends on the method of detection and ranges from 82% in FISH to nearly 94% in the reverse transcriptase polymerase

Table I. General characteristics of patients with synovial sarcoma

Patient	Female (f)/ Male (m)	Age (years)	Localisation (left/l, right/r)	Characteristics	Histological subtype	Diameter (cm)	% tumour necrosis after neoadjuvant treatment	Mean time without recurrence and metastasis (months)	Progression	Palliative treatment	Total time of follow-up (months)
KG	f	28	crus l*	primary	biph*	7	0	79	no	no	79
			crus l	after treatment	mono						
GK	m	28	shoulder l	primary	biph	13.5	5	12	yes	yes	33
			shoulder l	after treatment	biph						
			lung	metastasis	poorly						
			pericardium	metastasis	mono						
ED	f	32	knee l	primary	mono	20	75	49	no	no	49
			knee l	after treatment	mono						
SS	f	30	pelvis r	primary	mono	12	10	28	yes	yes	44
			pelvis r	after treatment	mono						
JŚ	m	37	foot l	primary	mono	8.5	5	54	no	no	54
			foot l	after treatment	mono						
MD	f	31	knee l	primary	biph	11	30	23	no	no	23
			knee l	after treatment	mono						
			knee l	recurrence	poorly						
MM	m	42	forearm r	primary	biph	5	30	36	no	no	36
			forearm r	after treatment	biph						
DP	f	44	arm r	primary	mono	4.8	10	27	no	no	27
			arm r	after treatment	mono						
EB	f	17	femur l	primary	biph	4	30	28	no	no	28
			lemur l	after treatment	biph						
HW	m	54	foot l	primary	mono	10	40	1	yes	yes	13
			foot l	after treatment	mono						

**biph – biphasic, mono – monophasic spindle-cell, poorly – poorly differentiated*

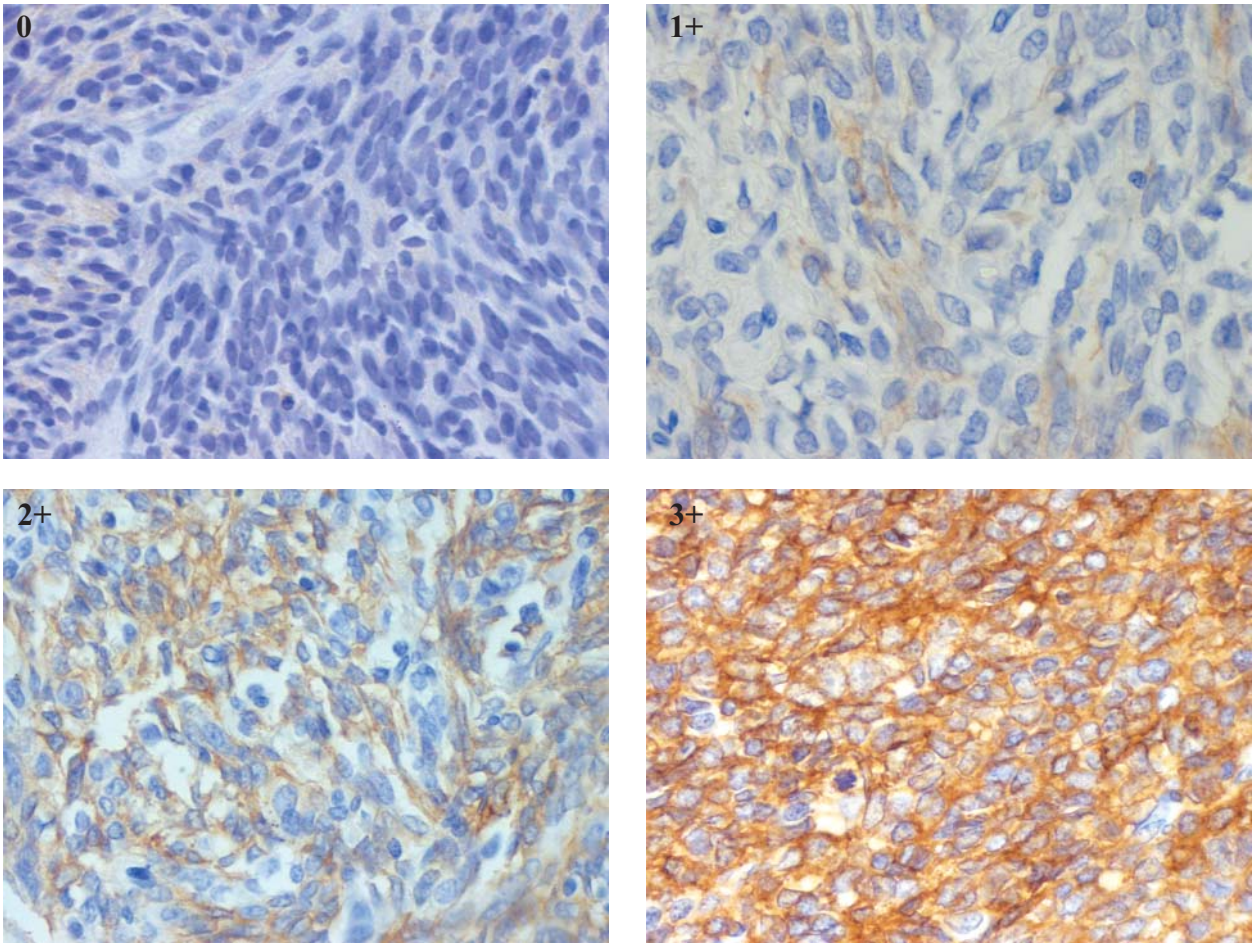

Fig. 1. Summary of the immunohistochemical evaluation of EGFR

chain reaction (RT-PCR) [19]. However, FISH is more available in pathology departments and is less expensive as well. Fresh or fresh frozen tissue is preferable in the RT-PCR testing. Formalin-fixed, paraffin-embedded tissue requires additional technical steps in the RT-PCR procedure. In addition there is a contamination effect associated with the PCR technology. A product of the translocation in SyS is a fusion (chimeric) protein group SYT-SSX. Most often, SYT-SSX1 (in over 60% of cases), SYT-SSX2 (about 40% of cases) and seldom SYT-SSX4 are found [20-22]. So far the biological function of these proteins has not definitely been characterized but most likely they play a key role in the oncogenesis of SyS. On the basis of research to date, the SYT-SSX fusion proteins may have an influence on particular stages of neoplastic transformation, e.g.: restoration of E-cadherin expression [23], promotion of cyclin D1 expression [24], promotion of p53 ubiquitinylation [25], interaction with transcription factor LIM homeobox protein 4 [26], induction of cell adhesion molecule claudin-7 expression [27], induction of insulin-like growth factor IGF-2 and CD44 [28, 29], and recruitment of β-catenin to the nucleus [30]. In one of the studies it was observed that the presence of the various SYT-SSX fusion protein types is not connected with a better overall prognosis, but there is an association with the histological subtype of SyS [20]. In our study all the cases had the t(X;18) translocation confirmed by

Table II. Summary of *EGFR*, *HER2* and *TOPIIA* FISH evaluation

	N*	Mean	Minimum	Maximum	SD
EGFR	22	1.94	1.42	2.90	0.36
CEP7	22	1.87	1.35	3.00	0.38
EGFR RATIO	22	1.04	0.92	1.16	0.07
HER2	22	2.35	1.58	4.41	0.82
CEP17	22	2.26	1.67	3.83	0.62
HER2 RATIO	22	1.02	0.88	1.33	0.10
TOPIIA	20	2.54	1.50	5.30	1.10
CEP17	20	2.19	1.40	3.62	0.68
TOPIIA RATIO	20	1.14	0.90	1.50	0.14

**N – number of cases*
***SD – standard deviation*

FISH. Three cases of biphasic SyS after treatment had an appearance of monophasic, spindle cell type. One of these cases subsequently showed the appearance of a poorly differentiated SyS in lung metastasis and a local recurrence. The morphological changes did not substantially influence the immunohistochemical or genetic profile of the tumour.

The results of studies on overexpression and amplification of HER2 in SyS differ substantially and frequently depend on the method used. Generally in the immunohistochemical staining from 10 to 35% positive results were obtained. Nevertheless, their comparison is problematic because of lack of uniform criteria of the assessment. In the studies with FISH methodology used, the percentage of cases with amplification was between 5 and 15%. The limited number of cases in the investigated groups still does not allow an objective statistical analysis [31-34]. In our study the majority of HER2 immunostainings of SyS before and after treatment were classified as negative or weakly positive (13%). The membranous reaction was the only taken into account. There was no evidence of amplification in the FISH study confirmed, when the standards of assessment established for breast cancer were implemented. Currently the overexpression of HER2 in epithelial neoplasms is thought to be associated with a higher grade and more aggressive course of the disease. However, in the mesenchymal tumours there is no full understanding of HER2 biological functions yet. Furthermore, it seems that cases of SyS, just the opposite to lung, bladder, large intestine or nasopharynx cancers, have better prognosis when associated with the *HER2* amplification. It has been identified in selected cases of thyroid cancer and osteosarcoma as well [35, 36].

Anthracyclines, including doxorubicin and epirubicin, are among the most widespread chemotherapeutic agents used in the treatment of both solid tumours and haematological malignancies [37]. The basic mechanism of their action is the inhibition of topoisomerase IIA, the enzyme that facilitates double-stranded DNA breaks and subsequently apoptosis. Another important mode of action of anthracyclines is the formation of free radicals occurring through membrane lipid peroxidation. This mechanism besides an antitumour effect has a strong cardiotoxic effect as well [37-40]. The anthracyclines have a low therapeutic index with frequent acute and subacute side effects including long-term treatment-related cardiomyopathy and secondary leukaemia. Administration of a potentially toxic drug to a patient requires preselection of individuals who may benefit most from this specific treatment. The gene coding *TOPIIA*, the primary target for anthracyclines, is located on chromosome 17 close to the *HER2* gene. The quantitative changes within *TOPIIA* can be identified by FISH. Numerous clinical studies have confirmed that patients with *TOPIIA* amplification within tumour cells have a significantly better response to anthracycline-based chemotherapy than patients with normal *TOPIIA* gene status [41-50]. About 25-35% of patients suffering from breast cancer have showed amplification or deletion in the *TOPIIA* gene (the latest trials of the Danish Breast Cancer Cooperative Group DBCG 89D and Breast Cancer International Research Group BCIRG) [50, 51]. The *TOPIIA* gene aberrations were mainly present among HER2 negative patients. Nevertheless, a relatively high proportion (22%) was identified among HER2 positive patients. In several studies the immunohistochemical evaluation of TOPIIA status, as in the case of EGFR, is not authoritative and the only recommended method is FISH. In chemotherapeutic schemes of SyS treatment anthracyclines are an alternative to ifosfamide. According to severe side effects of anthracycline-based chemotherapy, evaluation of *TOPIIA* status in FISH would be a key point for qualification of patients for this type of therapy. There are few studies on *TOPIIA* gene status in SyS [19, 31], mostly comprising single or series of cases; therefore objective conclusions cannot be formulated so far. In our study TOPIIA protein was strongly expressed immunohistochemically in 78% of cases, whereas there was no change in gene status detectable by FISH. In the one case of poorly differentiated synovial sarcoma the ratio for *TOPIIA* was 1.5 and may be classified as weakly positive. It can only be speculated that an aggressive course of malignancy of that particular patient was reflected in the *TOPIIA* amplification.

Most human neoplasms, particularly of the epithelial origin, are characterized by the functional activation of proteins which belong to the family of growth factors and their receptors. EGFR was the first growth factor receptor proposed as a target for cancer treatment [52, 53]. After more than 20 years of studies, currently four drugs with an antagonistic effect to EGFR are available. They are used in the treatment of metastatic epithelial cancers: non-small-cell lung carcinoma, squamous-cell carcinoma of the head and neck, colorectal and pancreatic carcinoma [54]. At present the EGFR antagonists are being tested on a wider group of neoplasms which only reveal the overexpression of EGFR protein. Chemotherapy with doxorubicin, ifosfamide, trabectedin, gemcitabine with docetaxel is used effectively in the treatment of SyS in combination with surgical resection

[55-57]. The activity of other drugs has remained disappointingly low in first-line chemotherapy, even though two vascular endothelial growth factor receptor-targeting agents, pazopanib and sorafenib, were reported to exert antitumour activity in advanced soft tissue sarcomas during the 2007 American Society of Clinical Oncology annual meeting [15, 57]. For targeted therapy blocking the pathways of signal transduction, four conditions have to be fulfilled: the target protein must be present and be in an activated form, it has to contribute to the oncogenic process, and it has to be inhibited efficiently by the drug used. Numerous investigations have confirmed the overexpression of EGFR in SyS [9, 32, 34, 58], but it has been impossible to prove absolutely its phosphorylated status and influence on oncogenesis. The *in vitro* studies show EGFR to induce proliferation of SyS cells and gefitinib to inhibit proliferation of the cell line, but only in very high concentrations [59]. Subsequent investigations have confirmed that such concentrations cannot be obtained *in vivo* [60]. Likewise, in non-small-cell lung carcinoma, colorectal or pancreatic carcinoma, strong immunohistochemical EGFR expression does not translate into a clinical answer to EGFR antagonists treatment. According to current recommendations immunohistochemical overexpression of EGFR cannot be the only method to identify patients qualifying for this type of therapy. The results of the initial studies on EGFR amplification in SyS were promising [31]. However, only a minority of the SyS cases show quantitative changes of the *EGFR* gene [61] in successive studies. Moreover, the mutations of EGFR within exons 18-21 which are associated with the response to *EGFR* antagonists treatment are rare; they comprise less than 15% of all cases and their function remains unclear [61, 62]. In our study, most of the cases of SyS showed overexpression of EGFR protein; immu-nohistochemical reaction was positive in 91% and strongly positive in almost 43% of cases. This high percentage of positive EGFR immuno-histochemical stainings was not accompanied by gene amplification tested by FISH. Virtually all cases of SyS showed no evidence of *EGFR* amplification and though it is postulated that EGFR contributes to the proliferation of SyS cells it seems that it does not play a central role in this model.

While our study was carried out the second phase of the clinical study with gefinitib therapy of SyS was started by the European Organization for Research and Treatment of Cancer (EORTC). The EORTC results were published in May 2008 and showed that the EGFR antagonists did not demonstrate sufficient activity and have minimal influence on the course of the disease [11].

In conclusion, our study revealed that the majority of SySs showed no evidence of HER2 protein overexpression or gene amplification; TOPIIA protein was strongly expressed immunohistochemically in 78% of cases, whereas there was no detectable change in gene status. Although most of the cases of SyS showed overexpression of EGFR protein, it was not accompanied by gene amplification.

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Address for correspondence

Anna Szumera-Ciećkiewicz MD
ul. Roentgena 5
02-781 Warsaw
phone +48 22 546 27 26
e-mail: annacieckiewicz@coi.waw.pl