Epidermal Growth Factor Receptor Expression and Activation in Nonseminomatous Germ Cell Tumors

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ABSTRACT

Purpose: The goal of this work was to study the expression of epidermal growth factor receptor (by use of monoclonal antibody EGFR 1) and HER-2/neu (by use of monoclonal antibody EGFR 2), as well as EGFR activation [phosphorylated EGFR (P-EGFR)] and autocrine stimulation [ligand transforming growth factor-α (TGF-α)] markers in a series of 24 testicular tumors [18 nonseminomatous germ cell tumors (GCTs), 1 Leydig cell tumor, and 5 seminomatous GCTs].

Experimental Design: Paraffin-embedded sections of tumors were studied immunohistochemically for β-human chorionic gonadotropin (β-hCG), EGFR 1, HER-2/neu, TGF-α, and P-EGFR expression. In one case of pure choriocarcinoma, fresh-frozen tumor sections were also evaluated. The presence of EGFR mRNA was studied in the Jar choriocarcinoma cell line using reverse transcription-PCR.

Results: Staining for cell membrane EGFR was detected immunohistochemically in the 16 β-hCG-positive components of 18 nonseminomatous GCTs as well as in the control Jar choriocarcinoma cell line and normal placenta. In contrast, 1 Leydig cell tumor, 5 seminomatous GCTs, and β-hCG-negative components of 18 GCTs, as well as control B and T lymphoma cell lines, did not express EGFR. Expression of HER-2/neu, TGF-α, and P-EGFR was detected in 25, 36, and 27% of EGFR-positive, nonseminomatous GCTs, respectively. EGFR mRNA was detected in the Jar choriocarcinoma cells.

Conclusions: We report data, for the first time, that document EGFR and HER-2/neu expression and indicate EGFR activation and autocrine stimulation in β-hCG-positive, nonseminomatous GCTs. These findings may be clinically relevant in relation to the recent availability of active EGFR- and HER-2/neu-targeted pharmaceutical agents and to the extensively described negative prognostic significance of β-hCG expression in mixed GCTs.

INTRODUCTION

In recent years, growth factors and growth factor receptors have been found to play an important role in the pathogenesis of several human malignancies. Among the best-studied growth factor receptors is EGFR, a 1 M, 170,000 plasma membrane glycoprotein composed of an extracellular ligand-binding domain, a transmembrane lipophilic segment, and an intracellular protein kinase domain with a regulatory COOH-terminal segment. After binding of ligand, EGFR dimerization occurs, which results in high-affinity ligand binding, activation of the intrinsic protein tyrosine kinase activity, and tyrosine autophosphorylation. These events lead to activation of a cascade of biochemical and physiological responses that are involved in the mitogenic signal transduction of normal as well as malignant cells (1). Several lines of evidence support the EGFR as a target for cancer therapy. EGFR expression is elevated in many epithelial tumors and tumor-derived cell lines, and coexpression of high levels of EGFR and its ligands lead to a transformed cellular phenotype. Furthermore, EGFR overexpression correlates with poor clinical outcome in a number of malignancies (2). Recently, novel classes of EGFR-targeted pharmaceutical agents, such as anti-EGFR monoclonal antibodies and tyrosine kinase small molecule inhibitors, have shown clinical activity against EGFR-expressing malignancies that are otherwise resistant to conventional chemotherapy and radiotherapy (3–9).

In a systematic search for malignancies expressing the EGFR (by use of monoclonal antibody EGFR 1) and HER-2/neu (by use of monoclonal antibody EGFR 2), potential therapeutic targets of anti-EGFR agents, we elected to study expression and activation of these EGFR family members in a series of 24 testicular tumors. In this report, we document, for the first time, that EGFR 1 and, in some cases, HER-2/neu membrane expression occurs in the β-hCG-positive component of mixed nonseminomatous GCTs, and that, in a fraction of patients, EGFR expression is associated with receptor activation and autocrine stimulation.

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3 The abbreviations used are: EGFR, epidermal growth factor receptor; GCT, germ cell tumor; β-hCG, β human chorionic gonadotropin; TGF, transforming growth factor; RT-PCR, reverse transcription-PCR; UPN, unique patient number; P-EGFR, phosphorylated-EGFR.
PATIENTS AND METHODS

**Diagnosis and Tumor Samples.** Characteristics of testicular tumors and serum β-HCG-positivity are reported in Table 1. All but two specimens were obtained from primitive testicular tumors. Biopsies of abdominal masses were performed in two cases of extragonadal primitive tumors (patients UPN 6 and UPN 7). In one case of pure extragonadal choriocarcinoma (patient UPN 6), fresh-frozen tissue sections from a liver metastasis were also evaluated. Histological diagnosis performed according to WHO criteria. Table 1. All but two specimens were obtained from primitive testicular tumors and serum β-HCG-positivity are reported in Table 1. All but two specimens were obtained from primitive testicular tumors. Biopsies of abdominal masses were performed in two cases of extragonadal primitive tumors (patients UPN 6 and UPN 7). In one case of pure extragonadal choriocarcinoma (patient UPN 6), fresh-frozen tumor sections from a liver metastasis were also evaluated. Histological diagnosis performed according to WHO criteria was by microscopic evaluation of 3–5 μm sections of paraffin-embedded tumor samples processed with H&E staining and by immunohistochemistry for β-HCG, cytokeratins, and placental alkaline-phosphatase expression. In particular, tumor β-HCG was detected by immunohistochemistry with a monoclonal antibody specific for the β-chain of HCG (β-HCG; Diagnostic BioSystem, Pleasanton, CA).

**Detection of EGFR, P-EGFR, HER-2/neu, and TGF-α by Immunohistochemistry.** EGFR, P-EGFR, and TGF-α expression was detected by indirect immunohistochemistry on paraffin-embedded tumor sections. EGFR was detected using a mouse monoclonal antibody specific for human EGFR (clone H111.6; NeoMarkers, Fremont, CA); the activated form of EGFR (P-EGFR) was detected using a mouse monoclonal antibody specific for tyrosine phosphorylated EGFR (clone 74; BD Transduction Laboratories, Lexington, KY); and TGF-α was detected using a mouse monoclonal antibody specific for native and some denatured forms of human TGF-α (clone 213-4.4; Oncogene Research Products, Cambridge, MA). Cell-bound antibody was then visualized by ChemMate AP Detection System (Dako, Gostrup, Denmark) consisting of a rabbit biotinylated antimouse antibody and streptavidin-labeled alkaline-phosphatase. EGFR 2 expression (for HER-2/neu) was detected by Dako HercepTest according to the manufacturer’s instructions (Dako, Gostrup, Denmark). In one case of pure choriocarcinoma (patient UPN 6), fresh-frozen tumor sections were also evaluated.

An immunoenzymatic double-staining procedure for β-HCG and EGFR was carried out on paraffin-embedded tissue sections. Briefly, after enzymatic digestion with Pronase (5 μg/ml) for 5 min at 37°C, tumor sections were incubated with anti-EGFR antibody and visualized by the nitroblue tetrazolium chromogen. Thereafter, the same sections were incubated with anti-β-HCG antibody and visualized by the red new fuchsin chromogen, obtaining a dual-color image with a blue cell membrane positivity for EGFR and red cytoplasmatic positivity for β-HCG. Expression of EGFR, P-EGFR, HER-2/neu, and TGF-α was also evaluated in the Jar human choriocarcinoma cell line and human placenta (positive controls) as well as in the EB-3 B-cell and Jurkat T-cell lymphoma lines (negative controls).

**Detection of EGFR mRNA by RT-PCR.** EGFR mRNA was evaluated in the Jar human choriocarcinoma cell line as well as in the EB-3 B-cell and Jurkat T-cell lymphoma lines and blood leukocytes from two healthy donors. Total cellular RNA was isolated from each cell pellet by using the RNAse Total RNA kit (Qiagen, Germany) according to the manufacturer’s instructions. After purification, the concentration and purity of

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* +, positive; –, negative; ND, not determined; Embr, embryonal carcinoma; Chorio, choriocarcinoma; Ter, teratoma; Endo, endodermal sinus tumor; Sem, seminoma.
RNA were analyzed by absorbance at 260 and 280 nm (Shimadzu model UV 160 A; Ref. 10). EGFR mRNA was detected by RT-PCR. Purified RNA (0.1 μg) was added to a reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 6.25 mM MgCl₂, 1 unit/μL RNase inhibitor, 1 mM deoxyribonucleoside triphosphates (dATP, dTTP, dCTP, and dGTP), 2.5 μmol/L of a 16-mer d(T)₁₆ oligonucleotide primer, and 2.5 units/μL Moloney murine leukemia virus reverse transcriptase in a total volume of 20 μL (all of the reagents were from Perkin-Elmer). The reaction was incubated for 30 min at 42 °C; 2.5 μL of this reaction mixture were added to a 25-μL reaction composed of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 0.2 mM deoxyribonucleoside triphosphates (dATP, dTTP, dCTP, and dGTP), 1.25 units of Taq Polymerase (Pharmacia Corp., Peapack, NJ), and 25 pmol of each primer. Primer sequences and PCR profile were used as described previously (11). The primers used for amplification had the sequences 5'-GAGAGAGAGAAGTCCGAGAGAGAGAGAGAG-3' and 5'-GTGACATTATCGAGAGAGAGAG-3'. The reactions were initially denatured at 94°C for 3 min. PCR amplification was performed in a Perkin-Elmer 9700 thermocycler with the amplification profile of 94°C for 1 min, 57°C for 30 s, and 72°C for 90 s. After 30 cycles of amplification, the PCR products were extended for 7 min at 72°C. The RT-PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide, and an image was saved by the GelDoc 1000 (Bio-Rad) system.

The RT-PCR amplification procedure was checked by addition of a fixed amount of RNA standard (1 × 10⁻¹⁹ mol) to the RT-PCR reaction described above. The RNA standard contained the same primer-binding sequences and was RT-PCR amplified with the same efficiency as the EGFR mRNA. The generation and characterization of the RNA standard has been described previously (11). The RT-PCR products derived from the EGFR mRNA and the standard RNA gave rise to bands of 454 and 314 bp, respectively.

RESULTS

Expression of EGFR (by Use of EGFR 1) and HER-2/neu (by Use of EGFR 2). Combined morphology and immunohistochemistry showed that in 16 of 18 nonseminomatous GCTs, EGFR (by use of EGFR 1) was strongly expressed in the β-HCG-positive component of mixed GCTs. In contrast, 1 Leydig cell tumor, 5 seminomatous GCTs, and β-HCG-negative components of 18 GCTs, as well as control B and T lymphoma cell lines, did not express EGFR. As shown in Fig. 1, dual-color immunohistochemistry confirmed the coexpression of membrane EGFR and cytoplasmatic β-HCG, with all β-HCG-positive cells coexpressing EGFR. Tumor β-HCG and EGFR expression corresponded to serum β-HCG positivity in all cases (Table 1). The same pattern of EGFR and β-HCG expression was found in the Jar choriocarcinoma cell line and normal placenta but not in the negative controls (healthy donor leukocytes and B- and T-cell lymphomas).

Coexpression of HER-2/neu (by use of EGFR 2) was found in 25% (3 of 12) of EGFR-positive nonseminomatous GCTs (Table 1 and Fig. 2). HER-2/neu was expressed on the cell surface membrane with the same tissue distribution as EGFR. The combination pattern of positivity differed in individual cases with EGFR-positive/HER-2/neu-positive (Fig. 2) and EGFR-positive/HER-2/neu-negative tumors (Fig. 3).

Evidence of TGF-α Ligand and P-EGFR. Intracellular expression of the EGFR ligand TGF-α was found in 36% (4 of 11) of EGFR-positive nonseminomatous GCTs (Fig. 3). TGF-α was expressed inside the cells with the same tissue distribution as EGFR.
Membrane expression of the phosphorylated form of EGFR (P-EGFR) was found in 27% (3 of 11) of EGFR-positive, nonseminomatous GCTs on archival paraffin-embedded sections. In one P-EGFR-positive case (patient UPN 6), a much more evident expression of P-EGFR was detected on fresh-frozen tissue sections (Fig. 3) rather than paraffin-embedded tissue sections. In the remaining 8 cases, a scattered positivity for P-EGFR was found. P-EGFR was expressed on the cell membrane at the edge of tumors (Fig. 3).

**Evidence of EGFR mRNA by RT-PCR.** The expression of EGFR was confirmed by detection of EGFR mRNA by RT-PCR in EGFR-positive choriocarcinoma Jar cells (Fig. 4). RNA (0.1 μg) isolated from Jar cells contained EGFR mRNA (Fig. 4A, Lane 6). In contrast, control human leukocytes and B- and T-cell lymphoma lines, which were EGFR negative by immunohistochemistry, contained no EGFR mRNA (Fig. 4A, Lanes 2–5, respectively).

To verify that RT-PCR amplification had taken place in all reactions, the RNA samples were also RT-PCR amplified in the presence of an internal RNA standard that was RT-PCR amplified with the same primers as the EGFR mRNA but produced a RT-PCR product of a smaller size (11). The internal standard RNA was detected in all of the reactions (Fig. 4B, Lanes 2–6, bottom band), demonstrating that RT-PCR amplification had occurred. However, only RNA from the Jar cells gave rise to a RT-PCR product from EGFR mRNA (Fig. 4B, Lane 6, top band). Marker DNA is shown in Lanes 1 and 7 (Fig. 4).
DISCUSSION

The recent availability of clinically active, EGFR-targeted pharmaceutical agents prompted us to evaluate EGFR 1 and EGFR 2 (HER-2/neu) in a series of 24 testicular tumors. In this report, we document for the first time that the β-HCG-expressing component of mixed GCTs is strongly positive for EGFR 1 with HER-2/neu coexpression in 25% of cases. HER-2/neu (EGFR 2) has been described as the preferred heterodimerization partner in the EGFR family. In contrast to the other members of the EGFR family, no fully characterized ligand binds directly to HER-2/neu, which probably acts as a common signaling subunit of all EGFRs, thus enhancing ligand affinity for the receptor. Interestingly, in experimental models coexpression of EGFR 1 and HER-2/neu is associated with a remarkable potentiation of mitogenesis and a poor prognosis (12). In our study a minority of cases coexpressed EGFR 1 and HER-2/neu. Whether these two phenotypic patterns, i.e., EGFR-positive/HER-2/neu-positive versus EGFR-positive/HER-2/neu-negative, correlate with different clinical behaviors, such as response to therapy and tendency to metastasize, remains to be determined.

Expression of the EGFR ligand TGF-α and the activated form of EGFR (P-EGFR) was found in 36 and 27% of EGFR-positive, nonseminomatous GCTs, respectively. TGF-α is an endogenous ligand able to induce EGFR-mediated cell activation, and it has been shown to be a potent promoter of angiogenesis. The concomitant expression of EGFR and TGF-α is described as a mechanism of cancer cell autocrine stimulation, resulting in a clinically aggressive disease and poor prognosis (2). Expression of P-EGFR lends further support to the possibility of EGFR-mediated cell activation playing a pivotal role in nonseminomatous GCT development, differentiation, and progression. This hypothesis is consistent with the recently described role of factors belonging to the epidermal growth factor and TGF-α families in the autocrine growth stimulation of embryonal carcinomas and undifferentiated teratocarcinomas in vitro (13–15). In our study, the detection of P-EGFR positivity may be underestimated because of the technical limitations of the antibody-binding capacity applied to archival paraffin-embedded sections.

EGFR is commonly expressed in epithelial cells, and its overexpression in malignant cells correlates with poor prognosis and, possibly, with response to anti-EGFR agents. Data presented in this report do not formally document the overexpression of EGFR because immunohistochemistry is not a reliable quantitative assay, whereas quantitative RT-PCR cannot be applied to mixed GCTs; in fact, in the case of mixed GCTs the specific EGFR mRNA derived from β-HCG-positive cells is diluted by mRNA derived from histological components negative for EGFR. Therefore, to rule out the possibility of illegitimate immunohistochemical expression of EGFR in β-HCG-positive components of mixed tumors, we elected to perform RT-PCR in a pure choriocarcinoma cell line immunohistochemically positive for EGFR. EGFR mRNA quantification in the Jar cell line was not been performed because no adequate normal counterpart could be found for comparative analysis.

GCTs can be cured by combined treatment with surgery and chemotherapy. Nevertheless, approximately one-third of patients with high-risk testicular cancer do not achieve durable remission with first-line chemotherapy, and salvage treatment with conventional or high-dose chemotherapy yields durable remission only in a minority of patients (16). Choriocarcinoma, rarely presenting as a pure disease, is the most aggressive component of mixed GCTs and is characterized by intense β-HCG expression, early hematogenous metastatic spread, and in some cases, resistance to available antitumor agents; its aggressive biological behavior reflects the capacity of its normal counterpart, the placenta, to invade blood vessels (17). In addition, choriocarcinoma may represent the final step of malignant transformation of a primitive embryonal carcinoma into a more metastasizing histotype, as suggested by the frequent detection of β-HCG-positive choriocarcinoma elements in metastases from primitive embryonal carcinomas; β-HCG production in association with embryonal carcinoma is considered the result of trophoblastic differentiation of the tumor (18). The presence of choriocarcinoma and embryonal carcinoma components in metastatic mixed tumors represents a negative prognostic factor for survival (19, 20) with a poor response rate to chemotherapy (21, 22) and a worse survival (23, 24) for patients with high-level β-HCG at the time of diagnosis and/or relapse.

Because of the above considerations and encouraged by the tumor response with the trastuzumab HER-2/neu-specific monoclonal antibody in a single case of GCT (25), the demonstration that β-HCG-positive, nonseminomatous GCTs possess targets of novel EGFR- and HER-2/neu-targeted pharmaceutical agents is expected to be relevant for designing clinical trials that aim to improve the therapeutic index for these malignancies.
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