Epidermal Growth Factor Receptor Protein Detection in Head and Neck Cancer Patients: A Many-Faceted Picture

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Abstract

**Purpose:** Epidermal growth factor receptor (EGFR) overexpression is associated with poor prognosis in head and neck squamous cell carcinoma (HNSCC). Despite intensive biomarker studies, a consensual method for assessing EGFR protein expression is still lacking. Here we set out to compare three EGFR detection methods in tumor specimens from HNSCC patients.

**Experimental Design:** Tumors were prospectively excised from a series of 79 high-risk HNSCC patients enrolled in a GORTEC-sponsored clinical trial. EGFR expression was determined using a ligand-binding assay on membranes, Western blotting (WB) on membranes and total homogenates, and immunohistochemistry (IHC) on tissue microarrays. In addition, phosphorylated EGFR (pEGFR) was measured by WB on membranes.

**Results:** Distributions and ranges of tumor EGFR expression were method dependent. Moderate positive correlations (Spearman coefficient \( r \approx 0.50 \)) were observed between EGFR expression measured by the binding assay and WB or IHC. pEGFR levels positively and significantly correlated with total EGFR expression measured by WB or ligand binding, but not by IHC. The highest correlation \( (r = 0.85) \) was observed between EGFR and pEGFR levels, both measured by WB on membranes. Interestingly, the fraction of phosphorylated receptor (pEGFR/EGFR both measured by WB on membranes) significantly declined with increasing tumor EGFR expression, by all assessment methods used.

**Conclusion:** This study shows significant correlations between EGFR detection methods. The observed relationships between EGFR and pEGFR indicate that high-throughput pEGFR/EGFR analyses merit further investigations and consideration for routine use in patient samples. 

**Imaging, Diagnosis, Prognosis**

**Introduction**

The epidermal growth factor receptor (EGFR) belongs to the receptor protein tyrosine kinase superfamily. Aberrant regulation of EGFR has been shown to promote multiple oncogenic processes, including EGFR-induced proliferation and repopulation of clonogenic cells, DNA repair following radiotherapy, as well as impairment of apoptosis and cell-cycle regulation (1). The EGFR is detectable in about 90% of head and neck tumors (2, 3). EGFR overexpression in head and neck squamous cell carcinoma (HNSCC) correlates with an aggressive phenotype (4, 5). In HNSCC-irradiated patients, EGFR overexpression is associated with poor prognosis (3) and increased responsiveness to accelerated radiotherapy (2, 6, 7). However, EGFR expression levels are not consistently correlated with treatment response. Given the lack of a reliable predictive value of EGFR expression as a marker of tumor responsiveness to EGFR inhibitors, EGFR measurements are no longer carried out in routine practice. Although EGFR-targeting drugs have proven clinical efficacy in HNSCC, a subset of patients do not benefit from EGFR inhibitors (8).

To date, correlative studies have yet to fully account for the observed relationship between EGFR overexpression and poor outcomes, or unravel the molecular mechanisms...
associated with treatment response. Possible causes for such discrepancy could involve EGFR detection by non-standardized methods used in clinical studies (see refs. 9, 10 for review). Among them, immunohistochemical staining of EGFR in paraffin-embedded tumor tissue is the most widely employed [e.g., in the Radiation Therapy Oncology Group (RTOG) 9003 study; refs. 3, 11], for either visual (12) or automated EGFR determination (11). 125I-EGF radio-receptor assays have also been used to quantify high-affinity EGF-binding sites in membrane preparations from human tumors (13). A simplified binding assay was shown to correlate well with the full Scatchard analysis, thus proving more amenable to routine practice (14). Finally, immunodetection of EGFR by Western blotting (WB) is another method for assessing EGFR protein levels. WB analysis not only allows quantification of protein levels but also the detection of specific phosphorylation events indicative of receptor activation and alterations in the apparent molecular weight of the protein (e.g., resulting from alternative splicing or gene mutation or posttranslational modifications). Although this technique is routinely used for basic research, it is less frequently applied in the clinical setting.

Elevated EGFR levels in HNSCC have primarily been attributed to enhanced transcriptional activity (15), rather than to amplification of the EGFR gene. The EGFR gene displays different polymorphisms, which have been suggested to participate in the regulation of EGFR expression (16, 17). These polymorphisms include a polymorphic dinucleotide repeat (9–23 CA) in intron 1 and 2 single-nucleotide polymorphisms (SNP) located in the promoter region at positions −216 (G>T) and −191 (C>A).

The main objective of this study was to elucidate the relationship between EGFR protein detection methods in a series of postoperative HNSCC in the clinical setting. We compared EGFR expression using the ligand-binding assay, immunodetection by immunohistochemistry (IHC) and WB. The influence of EGFR polymorphisms on EGFR expression was also examined.

Materials and Methods

Patient tumor samples

Seventy-nine HNSCC samples were obtained from patients eligible for biologic collection before inclusion in the CARISSA multicenter blinded Institutional Review Board–approved phase II trial of postoperative irradiation with cisplatin ± gefitinib (GORTEC 2004-02-106 NCT00169221, RJB Principal Investigator). The Carissa trial, designed to include 140 patients, was closed early as a result of slow accrual subsequent to the IMEX and Bonner studies (8, 18). As a result, only 79 patients were included in the biomarker study. Inclusion criteria required tumor samples to contain at least 50% tumor cells, determined by hematoxylin–eosin (H&E) saffron or (H&E) staining of an apposition smear on a portion of fresh tumor (≥0.5 cm²) excised from the operative specimen. Biologic data were reported in accordance with the quality recommendations for reporting on tumor markers (REMARK criteria) used in prognostic studies (19). Half of the tumor fragment (mean = 190 mg) was frozen in liquid nitrogen within 15 minutes after surgery and subsequently processed for use in quantitative WB and binding assays and for determination of EGFR polymorphisms (Fig. 1). The remaining mirrored tumor fragment was used for histologic control and fixed in formalin for immunohistochemical analysis. Frozen tumor samples were homogenized and membranes prepared as previously described (14), with addition of protease inhibitors (EDTA-free protease inhibitor cocktail tablet) and phosphatase inhibitors (10 mmol/L sodium pyrophosphate, 5 mmol/L sodium orthovanadate, and 100 mmol/L NaF) from Sigma-Aldrich.

Translational Relevance

Epidermal growth factor receptor (EGFR) protein expression has a strong prognostic value in head and neck squamous cell carcinoma (HNSCC), yet expression levels are not consistently correlated with treatment response. EGFR levels can be determined by quantitative and semiquantitative methods. However, a standard reference measurement for clinical application is lacking to date. We determined EGFR and phosphorylated EGFR (pEGFR) levels using 3 different techniques ([125I]-EGF binding, Western blotting (WB), and immunohistochemistry) in 79 tumor samples from a prospective series of poor-prognosis HNSCC patients. Positive interassay correlations were observed between all 3 methods but distribution profiles were different. The strongest positive correlation was observed between levels of pEGFR and total EGFR determined by WB on membrane preparations. Interestingly, the higher the total EGFR expression, the lower the fraction of phosphorylated receptors. Our results reveal tight EGFR expression–phosphorylation coupling in HNSCC with still-to-be-identified negative regulatory mechanisms that may impact treatment response.
Binding assay
The number of EGF-binding sites was determined as previously described (14) on membrane preparations using human recombinant EGF as the ligand. The limit of sensitivity was 1 fmol/mg protein. Coefficient of variation for interassay variability, computed on 7 series of analyses, was 18%, using a placenta membrane pool as control (mean 210 fmol/mg of protein).

Immunohistochemistry on tissue microarrays
Representative carcinoma areas were selected on hema-toxylin–phloxin saffron–stained sections. Three tissue cores (0.6 mm in diameter) from patient paraffin blocks were transferred into recipient blocks using a tissue microarray (TMA) Beecher Instrument Inc. workstation. Six normal tissue cores per TMA were included as technical controls. Membrane EGFR expression was assessed on triplicate TMA spots using the anti-EGFR antibody 3C6 (Ventana, Roche) and ultraview 3,3’-diaminobenzidine detection kit in a Benchmark XT (Ventana, Roche) autostainer. Samples were deemed unsuitable for analysis when cores were missing or when no tumor cell was found. A HNSSC slide overexpressing EGFR (positive control) was tested in the same staining run as TMA slides; omission of the primary antibody served as negative control. Immunohistochemical slides were scanned and evaluated by 2 pathologists blinded to each other’s scoring. EGFR staining intensity was scored as: 0 = no staining; 1+= weak staining; 2+= moderate staining, and 3+= strong staining. The quick score (QS) was defined as the percentage of stained tumor cells multiplied by the staining intensity (0, 1, 2, or 3). QS ranged between 0 and 300 arbitrary units. Analyses were done on mean QS obtained from triplicate TMA cores. For assessment of interobserver reproducibility, the data were compared and slides were reviewed by both pathologists in case of major discrepancies (>10%).

Immunohistochemical staining with the same phosphorylated EGFR (pEGFR) antibody as that used for WB (Cell Signaling) showed a cytoplasmic granular pattern, which could not be extinguished by overnight phosphatase treatment. This staining was thus considered as nonspecific and pEGFR was not assessed by IHC.

Western blot
Prior to analysis of patient tumor samples, WB conditions were optimized (quantity of starting material and antibody selection) using the CAL33 human HNSCC cell line (20) and frozen tumor samples. Specific detection of total and tyrosine phosphorylated EGFR (21) was done using rabbit anti-EGFR (ref 2232) and anti-pEGFR Y1068 (ref 2234) from Cell Signaling. Secondary antibodies coupled to horseradish peroxidase were from Jackson Immunoresearch Laboratories.

Tumor samples (homogenates or membranes) and controls were subjected to SDS-PAGE on 7% acrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon-P Transfer Membrane; Millipore). Immune complexes on membranes were detected by enhanced chemiluminescence (Pierce). Signal intensity was visualized by autoradiography. Chemiluminescence was quantified using the GeneGnome Bio Imaging System (Syngene), allowing a wide linear response range (grey scale from 1–65,500). Interassay variability calculated on control values from 6 independent runs was 26% and 27% for membrane EGFR and pEGFR, respectively. Quantitative data were normalized to control values within each series of 6 to 8 patient samples. Controls corresponded to homogenates or membranes prepared from exponentially growing CAL33 cells treated for 5 minutes with 30 ng/mL EGF. Cell membranes were prepared by hypotonic lysis and high-speed centrifugation. Actin staining was done to evaluate the integrity and cellularity of the tumor samples.

EGFR polymorphisms
Analysis of the CA repeat polymorphism in intron 1 (rs 11568315) was done by fluorescent genotyping on Beckman CEQ 800 on genomic DNA extracted from tumors, as previously described (17). According to previous studies of EGFR gene polymorphisms, 17 CA repeats was chosen as a discriminatory value between short and long CA repeats (22). Two polymorphisms located in the promoter region, namely −191 C>A (rs 712829) and −216 G>T (rs 712830), were analyzed by PCR-RFLP, as previously described (23).

Statistics
Correlations between EGFR quantitative data were tested by means of the nonparametric Spearman correlation test. Relationships between EGFR polymorphisms and EGFR quantitative data were analyzed using the Fisher exact test. Statistics were carried out using the SPSS v15 software.

Results
Tumors from 79 high-risk head and neck cancer patients were included in this study. The flowchart of sample processing for biologic analyses is shown in Fig. 1, as well as the number of tumors analyzed in each assay. Seventy-eight tumors were available for IHC. Ten frozen samples were excluded for technical reasons (time-to-freezing ≥ 15 minutes). Hence, tumor homogenates and membranes were obtained from 69 patients. WB was done on 69 total homogenates and 60 membrane preparations. Sixty-six membrane preparations were suitable for binding assays, and DNA extracted from 66 tumors was examined for EGFR gene polymorphisms.

Immunohistochemistry on tissue microarrays
Immunostainings of representative carcinomas and their corresponding QS are presented in Fig. 2. QS (median 150, range: 0–300) showed a symmetrical distribution (Fig. 3). Of the 78 patients analyzed, only 2 were null for EGFR expression by IHC. Cytoplasmic immunoreactivity was not taken into account and nuclear EGFR staining was not detected.
Western blot analysis

Results from all WB analyses of EGFR or pEGFR in tumor membranes are shown in Figs. 3 and 4. Visual examination of WB autoradiograms reveals a wide range of EGFR and pEGFR levels in tumor membranes. Quantification by chemiluminescence imaging allows accurate measurements and extremely sensitive detection of even the faintest bands, as opposed to densitometric scanning of autoradiograms. EGFR and pEGFR were measurable in all but 2 of the tumor membrane preparations (Fig. 4), and EGFR was detected in all but one of the tumor homogenates (data not shown). Levels of EGFR expression were extremely high in some tumors (A19, E1, EX4, E8, D5, AX6, and D6), with values comparable with those observed in membranes prepared from the EGFR-overexpressing cell line. Using an antibody against a cytoplasmic epitope of EGFR, we detected no faster migrating bands in the range examined (125–200 kDa) that could correspond to the 145-kDa EGFR variant III (EGFR-vIII; ref. 24).

Binding assay

A wide range of EGFR expression levels was also observed with the binding assay on tumor membranes. EGFR
expression was detectable in all samples (3 being excluded for insufficient protein quantity). The median value was 264 fmol/mg of protein (range: 28–3,814).

Correlations between different detection techniques

Figure 3 illustrates the distribution of EGFR expression according to the different methods tested (binding assay,
by analyzing the pEGFR to EGFR ratio, both measured by WB. The fraction of phosphorylated receptor was estimated by WB on homogenates and membranes. Not surprisingly, a significant correlation was observed between EGFR levels measured by IHC and WB on total homogenates or membranes. A very strong positive correlation was observed between EGFR and pEGFR, both measured by IHC, or WB on total homogenates or membranes. With the exception of IHC (QS data), which exhibited a more bell-shaped curve, all measurements showed an asymmetrical distribution.

Figure 5 summarizes the correlations between the different analytical methods. For each method, N indicates the number of tumors analyzed. Significant correlations are represented in bold characters ($P < 0.05$) and the corresponding line thickness is related to the strength of the correlation. Nonsignificant correlations (NS) are indicated with dashed lines.

IHC, or WB on total homogenates or membranes. With the exception of IHC (QS data), which exhibited a more bell-shaped curve, all measurements showed an asymmetrical distribution.

Figure 5 summarizes the correlations between the different parameters analyzed. Not surprisingly, a significant correlation was observed between EGFR levels measured by WB on homogenates and membranes ($r = 0.67$, $P < 0.001$). The number of EGFR-binding sites correlated moderately well with EGFR levels measured by WB on membranes ($r = 0.50$, $P < 0.001$), or total homogenates ($r = 0.58$, $P < 0.001$), and with pEGFR measured by WB on membranes ($r = 0.50$, $P < 0.001$). A moderate correlation was also observed between IHC and WB on total homogenates ($r = 0.50$, $P < 0.001$). The weakest significant correlation was observed between EGFR measured by IHC and WB on membranes ($r = 0.29$, $P = 0.022$). No significant correlation was observed between EGFR expression determined by immunohistochemical analysis and pEGFR levels measured by WB on membranes.

A very strong positive correlation was observed between EGFR and pEGFR, both measured by WB on membranes, as indicated in Fig. 5 ($r = 0.83$, $P < 0.001$) and illustrated in the histogram of Fig. 6A. Importantly, the data do not suggest any saturation phenomenon, even at the highest levels of EGFR expression (Fig. 6B). However, they indicate that pEGFR increases only 3-fold when total EGFR increases 10-fold (determined following log10 transformation, Fig. 6B). The fraction of phosphorylated receptor was estimated by analyzing the pEGFR to EGFR ratio, both measured by

\[
\text{log}_{10} \frac{p\text{EGFR}}{\text{EGFR}} = 0.49 \times \log_{10} \text{EGFR} - 0.56.
\]

$P$ value was less than 0.001 and $r = 0.81$ ($N = 60$). The slope at 0.48 indicates that pEGFR increases 3-fold ($10^{0.48}$) when total EGFR increases 10-fold ($10^{0.08}$). C, log–log plot of the computed pEGFR/EGFR ratio versus total EGFR, both measured by WB on membranes. Following log10 transformation of the data, the equation for linear regression (Gaussian distribution) was the following:

\[
\text{log}_{10} \frac{p\text{EGFR}}{\text{EGFR}} = \left(\log_{10} \frac{1}{p\text{EGFR}}\right) - \text{constant}.
\]

$P$ value was less than 0.001 and $r = 0.82$ ($N = 60$).
With regard to the distributions of EGFR expression observed by binding or WB, results from previous studies showed a similar leftward-shifted distribution using a binding assay on tumor biopsies from HNSCC (5) and glial tumors (26). In contrast with IHC, methods that involve EGFR measurement in tumor homogenates and/or membranes (i.e., WB and binding) cannot discriminate between EGFR expression in tumor and stromal cells. Stromal cells, including fibroblasts, immune cells, and endothelial cells, exhibit null or low EGFR levels that may "dilute" the signal. Thus, for a given quantity of protein, samples rich in tumor cells, with moderate EGFR expression and few stromal cells, may be quantitatively similar to samples with fewer, highly overexpressing tumor cells and a larger stromal compartment. Importantly, patient samples analyzed in this study contained at least 50% tumor cells, thus limiting this dilution effect. In addition, sample heterogeneity in noncellular components, such as collagen fibers, fibronectin, and other extracellular proteins within tumors may contribute to variability in EGFR expression levels between tumors. Furthermore, compared with a full analysis of paraffin blocks, histospots on TMA might not be entirely "representative" of the entire tumor. Thus, visual assessment by a pathologist may target tumor cell staining in an operator-dependent way (11). Although this may be an advantage for tumor cell EGFR detection, there is no uniform scoring system (12), and the interpretation of staining intensity remains somewhat subjective (9). In addition, unlike quantitative WB and binding assay measurements, semiquantitative IHC may underestimate the quantity of EGFR, when super-saturating levels of EGFR are expressed and thus affect the distribution curve.

With regard to the moderate-to-low correlation observed between EGFR quantification by WB and IHC, the above-mentioned stromal contributions to WB analyses may also be involved. The low concordance between results from IHC and WB could be accounted for in part by the use of different antibodies. Significant differences have been shown in IHC analyses done with different antibodies (12, 27). Also, WB analysis does not distinguish between intracellular EGFR and cell surface EGFR, whereas Immunohistochemical quantification can specifically address intracellular staining visually. Because IHC scoring only takes into account cell surface EGFR, intracellular pools (i.e., endosomal and nuclear compartments) may be underestimated (28).

The strongest positive correlation was observed between the levels of total and pEGFR determined by WB in membranes. Hence, the highest levels of phosphorylated receptors were found in tumors with the most abundant EGFR expression. From the significant relationship illustrated in Fig. 6, there is no tendency toward a saturation process. Among the 29 EGFR phosphorylation sites (http://www.uniprot.org/uniprot/P00533), we chose to analyze tyrosine residue 1068. This phosphorylation site is in the C-terminal SH2/P1B interaction domain and activates crucial signaling pathways such as the mitogen-activated protein kinase and B-Raf pathways such as the mitogen-activated protein kinase and B-Raf pathways.
Akt pathways. Although the use of a single phosphorylation site on Y1068 may have limitations, this site is of clinical relevance, as shown by the marked inhibition of receptor phosphorylation by the clinically validated EGFR inhibitor gefitinib at Y1068 (29, 30). Overall, the finding that pEGFR levels correlate strongly with EGFR expression suggests that there is no major rate-limiting regulatory step leading to receptor phosphorylation, at least at Y1068. This observation is in line with the notion that EGFR overexpression is the principal mechanism of EGFR activation in various malignant tumors. However, EGFR tyrosine phosphorylation is complex and its regulation can occur at multiple levels including ligand-induced dimerization and receptor activation, internalization, recycling, and dephosphorylation (1, 31). Indeed, tumors with a high density of receptors have an enhanced capacity to dimerize and exhibit sustained phosphorylation independently of the ligand (32). Of note, receptor clustering may also be favored by physical parameters, such as membrane fluidity (33), that determine the potential of receptors to homodimerize (32). Other mechanisms of sustained EGFR activation in tumors include the presence of an autocrine loop with ligands such as amphiregulin (34) and endosomal signaling through the Ras pathway. In addition, 2 main processes of negative control have been identified, namely, dephosphorylation of key residues by phosphatases and removal of surface receptors by endocytosis. Tyrosine phosphorylation is regulated by tyrosine-specific phosphatases that reverse tyrosine phosphorylation and shut down signaling. For example, the protein tyrosine phosphatase kappa has been shown to regulate both basal ligand-independent and ligand-activated EGFR tyrosine phosphorylation at Y1068 in keratinocytes (35). Receptor internalization and degradation represent another level of negative regulation (36–39).

Interestingly, our data have led us to make novel hypothesis-generating observations about the fraction of phosphorylated EGFR observed in tumors from HNSCC patients. Even though the pEGFR/EGFR ratio does not reflect the real fraction of phosphorylated EGFR molecules, this computed ratio of arbitrary units allows 2 statements to be made. First, there is a 36-fold variability in the ratio of pEGFR to total EGFR measured by WB on membranes. Second, the fraction of phosphorylated receptors decreases when total EGFR expression increases, by all detection methods (illustrated in Fig. 6 for EGFR measured by WB on membranes). This negative correlation between the pEGFR/EGFR ratio and total EGFR can be explained by the fact that pEGFR increases by only 3-fold when total EGFR increases by 10-fold. Together these observations may reflect some yet-to-be-defined negative retrocontrol mechanisms which are induced by the pathways that they attenuate (40). This original observation remains to be evaluated in a preclinical HNSCC model and further confirmed in an independent clinical validation set.

Another strong correlation occurred between EGFR measured by WB on membranes and in total homogenates. This finding could validate the use of tumor extracts rather than membranes for EGFR detection and thus may simplify sample handling and reduce the tumor volume required for analysis. Also, the binding assay was significantly correlated with WB analyses (membranes and homogenates) and with IHC. This concurs with our previous findings showing a positive association between the ligand-binding assay and IHC (13).

Although the cytoplasmic-directed antibodies used in this study for WB EGFR and pEGFR analyses should recognize the truncated EGFRvIII species, we did not detect proteins with electrophoretic mobility around 145 kDa, suggesting the absence of EGFRvIII in the patients studied. The EGFRvIII is a truncated receptor that has lost most of the extracellular ligand binding, thus resulting in a constitutively active receptor and ligand-independent resistance to anti-EGFRs (24). As immune approaches that target EGFRvIII are currently being developed (41), more in-depth studies of EGFR expression in HNSCC would be of interest.

EGFR gene polymorphisms were also examined in this study as a potential indirect determinant of protein expression (16, 17). This study did not confirm a link between EGFR expression by IHC, WB, or binding, and EGFR intron 1 polymorphism. This contrasts with our previously reported study on HNSCC cancer patients (17), although it is consistent with a recent in vitro study conducted on 24 cell lines, including 12 derived from head and neck tumors (42). It is thus hard to draw a definitive conclusion about the relationship between intron 1 polymorphism and gene expression in HNSCC.

In conclusion, this study clearly illustrates the multi-faceted picture of EGFR expression in HNSCC. The present results highlight the current challenges for establishing standardized detection methods to enable more reliable assessments in clinical trials and applicability to routine practice, as well as to further investigate the role and regulation of EGFR in the oncogenic process. The binding assay has the advantage of quantifying functional receptors. However, it may prove rather difficult to obtain this measurement in routine practice as it consumes bench time and requires relatively large quantities of tumor tissue and radioactive reagents. WB is a sensitive detection method for quantification of total, phosphorylated, and truncated/cleaved receptor levels. However, like the binding assay, it is labor intensive. Given the stromal components intrinsically present in tumor homogenates, IHC seems more appropriate for routine EGFR detection than WB or binding assays. Indeed, several teams have switched to automated immunohistochemical analysis (11, 43). It may be projected that automated IHC will extend to routine practice, provided that automated methods are developed to properly account for tumor heterogeneity (i.e., distinguish between tumor cells vs. stromal components by virtue of scrupulous cut-and-paste definition of tumor cell areas). Intracellular EGFR, as opposed to cell surface, may also be worth quantifying in the light of recent findings that pinpoint the biologic importance of intracellular signaling in tumor cells.
(31) and possible therapeutic implications (44). Finally, determining pEGFR together with EGFR levels may provide important clues to regulatory mechanisms operating in HNSCC. The recent development of novel methodologic tools for EGFR and pEGFR detection, and identification of the specific residues phosphorylated (e.g., antibody array technology, multiplex assays, and isoelectric focusing technology), should encourage such analyses on clinical samples, with the ultimate goal of selecting patients for anti-EGFR-targeted therapies.

Disclosure of Potential Conflicts of Interest
R.J. Bernadouin is on the Amgen and Novartis Advisory Boards. The other authors disclosed no potential conflicts of interest.

References


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