Gain of allelic gene expression for IGF-II occurs frequently in Barrett’s esophagus

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Barrett’s esophagus involves the accumulation of genetic alterations that endow the cells with certain growth advantages (22). In metaplastic Barrett’s cells, such advantages could result from alterations in the expression of growth factors and their receptors. The binding of growth factors to receptors that are members of the protein tyrosine kinase (PTK) family [e.g., EGF receptor (EGFR)] can promote cellular proliferation by activating mitogenic signal transduction cascades. In Barrett’s esophagus, cellular levels of EGFR (also called Erbb1) and its ligands, EGF and TGF-α, have been found to be increased both in the metaplastic epithelium and in adenocarcinomas. This suggests that growth factors and their receptors play a role in the neoplastic progression of Barrett’s esophagus (2, 11).

The IGF-I receptor (IGF-I-R), a tetrameric glycoprotein with intrinsic PTK activity, was recently implicated in the neoplastic progression of Barrett’s esophagus (10). IGF-I-R is located on the surface of almost every cell (1). Ligand binding stimulates receptor autophosphorylation, effecting intrinsic PTK activity that activates signaling cascades regulating the expression of genes involved in cellular proliferation, differentiation, or apoptosis (17, 19). In a recent study, IGF-I-R expression was found in nondysplastic Barrett’s epithelium, and the level of expression was found to increase as dysplasia progressed in severity (10). Accordingly, the highest IGF-I-R levels were found in esophageal adenocarcinomas and in their associated lymph node metastases. Those data suggest that alterations in the IGF pathway may be early events in the neoplastic progression of Barrett’s esophagus. However, that study did not investigate the role of IGF-II, an IGF-I-R ligand that has potent proproliferative and antiapoptotic effects that appear to contribute to the development of a number of human tumors (25).

The IGF-II gene normally exhibits genomic imprinting, a reversible modification of DNA that allows gene expression from only one of the inherited alleles (8). Loss of imprinting (LOI) of the IGF-II gene results in a gain of allelic gene expression (GOAGE) by permitting IGF-II expression from both alleles, thereby increasing IGF-II production to levels that might contribute to carcinogenesis. Indeed, GOAGE for IGF-II has been shown to stimulate cell growth and prevent apoptosis through signaling via IGF-I-R (15). GOAGE for IGF-II was

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first reported in childhood tumors such as Wilms’ tumors but has since been found in adult tumors, including hepatocellular, gastric, colonic, and esophageal squamous cell carcinomas (3, 5, 13, 14, 28). GOAGE of IGF-II has also been found in adenomatous polyps in the colon, an observation suggesting a role for the growth factor early in the process of neoplastic progression (5, 27). We have studied GOAGE for IGF-II in the gastric, colonic, and esophageal squamous cell carcinomas (3, 5, 13, 14, 28). GOAGE of IGF-II in Barrett’s esophagus

**MATERIALS AND METHODS**

*Patients and tissue samples.* Patients with biopsy-verified Barrett’s esophagus who were scheduled for endoscopic surveillance at the Dallas Veterans Affairs Medical Center were invited to participate in the study. A total of 43 patients with Barrett’s esophagus were enrolled, including 31 patients with long-segment Barrett’s esophagus (≥3 cm of esophageal specialized intestinal metaplasia), 11 patients with short-segment Barrett’s esophagus (<3 cm of esophageal specialized intestinal metaplasia), and one patient whose length of Barrett’s esophagus was not determined. Surveillance biopsy specimens from 39 patients exhibited no histological evidence of dysplasia or cancer, 2 patients had low-grade dysplasia, and 2 patients had high-grade dysplasia or cancer. Written informed consent to participate in the study was obtained from all patients. Biopsies of Barrett’s esophagus, gastric body, and duodenal epithelia were obtained using a jumbo biopsy forceps (Olympus FB-50K-1), immediately snap-frozen, and subsequently stored at −80°C. For RNA extraction, samples were placed in RNA later solution (Ambion, Austin, TX) and stored at 4°C. The study was approved by the Institutional Review Board at the Dallas Veterans Affairs Medical Center.

*DNA and RNA isolation.* In biopsy samples from the first 25 patients, DNA were extracted using phenol chloroform and RNA were extracted using TRIzol reagent as previously described (21, 23). Technically simpler extraction procedures were used for the biopsy samples from the remaining 18 patients [DNA were extracted using the RNeasy mini kit protocol (Qiagen, Valencia, CA) and RNA were obtained using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions]. The DNA and RNA were quantitated using spectrophotometry. Total RNA were treated with RNase-free DNase (Ambion) in accordance with the manufacturer’s instructions to remove any contaminating genomic DNA (gDNA). cDNA were synthesized using equal amounts of DNA-free total RNA and 100 ng of random primer using SuperScript II RT (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. As a control, the reaction was performed on DNA-free total RNA in the absence of RT.

*Identification of IGF-II genomic polymorphism.* Allelic heterozygosity in IGF-II was determined by performing PCR amplification followed by restriction enzyme digestion or by performing allele-specific PCR amplification using the AplI restriction enzyme site in exon 9 of the IGF-II gene on gDNA. For biopsy samples from 22 patients, PCR was performed in 50 μl of reaction mixture containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 1 mM 2-deoxynucleotide 5′-triphosphate (dNTP), and 500 nM primers (sense primer, 5′-GGGTTGTTGCACTTATTTGCCA-3′; antisense primer, 5′-GGGTTGTTGCACTTATTTGCCA-3′). These primers were designed to amplify a segment of IGF-II containing an AplI polymorphism. Aliquots (1 μl) of DNA were added to the reaction mixture. Initial denaturation was performed at 95°C for 10 min, followed by 37 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. AmpliTaq Gold (1.25 U; Applied Biosystems, Foster City, CA) was used in the “hot start” PCR. Amplified DNA were electrophoresed on agarose gels. IGF-II DNA PCR products were digested overnight using AplI restriction enzyme (Roche Molecular Biochemicals, Indianapolis, IN). After digestion, PCR products were again electrophoresed on 6% polyacrylamide gels and visualized using ethidium bromide staining. Informative samples were identified by the presence of three distinct bands after gel electrophoresis (20).

For the remaining DNA from 12 patients, the technique of allele-specific PCR was performed as previously described (26). Briefly, we used allele-specific 20-mer sense primers designed to anneal with the 3′-end to either the intact AplI site at nt 820 (HSIGF-II/GenBank accession no. X07868) primer Y4 (5′-CAGCAAAGAGAAGAAAGAGAG-3′) or to the corresponding AplI polymorphism primer N2 (5′-CAGCAAAGAGAAGAAAGAGAT-3′) (26). Primer specificity for either allele was determined on the basis of the last nucleotide at the 3′ position of the primers, which corresponds to the polymorphism (bold), and allele specificity was improved by the introduction of a nt mismatch at position 1 at the 3′-end of the primer (underlined) (26). Both alleles were amplified using the same antiserum primer B (5′-GGGTTGTTGCACTTATTTGCCA-3′). PCR was performed in a total volume of 50 μl for 37 cycles consisting of denaturation at 95°C for 1 min, annealing at 53°C for 30 s, and extension at 72°C for 30 s. Each reaction contained 10 ng of genomic DNA, 2.5 μM primer (12.5 pmol total), 1.7 U of AmpliTaq Gold (Roche Applied Biosystems, Indianapolis, IN), and standard amounts of MgCl2, PCR buffer, and dNTP. PCR products were then electrophoresed on 2% agarose gels and stained using ethidium bromide. Samples informative for AplI polymorphism were identified using a PCR product resulting from primers Y4 and B (alleles containing the polymorphism designated allele A) and primers N2 and B (alleles not containing the polymorphism designated allele B) corresponding to each of the two IGF-II alleles (26). In a subset of patients, we performed the AplI PCR followed by the AplI digestion method in addition to using allele-specific PCR and found that these techniques yielded identical results. The remainder of the study was therefore performed using the far more efficient method of allele-specific PCR.

*Allele-specific gene expression using exon connection PCR.* After identifying the informative samples as described above, corresponding cDNA were then amplified to assess for monomorphic or biallelic mRNA expression of IGF-II using a nested exon connection RT-PCR as described previously (26). The nested exon connection strategy was used to minimize the interference of small amounts of remaining gDNA in the cDNA preparation in the PCR.

In the first PCR, a cDNA-specific primer pair that spanned the boundaries between exons 8 and 9 of IGF-II was used as described previously (26). The sense primer 2 (5′-CTTCGGACCTTGGTCTCG-3′) and the antisense primer 2 (5′-CTTCGGACCTTGGTCTCG-3′) were used to amplify a fragment of 110 bp cDNA fragment of the IGF-II gene (26). Each 50 μl of PCR product contained 1 or 5 μl of cDNA (corresponding to 25 or 125 ng of total RNA), 2.5 μM concentrations of each primer (12.5 pmol total), 1.7 U of AmpliTaq Gold (Roche Applied Biosystems), and standard amounts of MgCl2, PCR buffer, and dNTP. PCR was begun with 5-min predenaturation at 95°C, followed by 37 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

In the nested secondary PCR, a sense primer A (5′-CTGGACTCCAAAAATTGGC-3′) and the antisense primer B described above were used to amplify a 293-bp fragment of exon 9 of the IGF-II gene, which included the AplI site (26). The 50-μl reaction mixture contained 1 μl of the PCR exon connection PCR product, 2.5 μM concentrations of each primer (12.5 pmol total), 1.7 U of AmpliTaq Gold (Roche Applied Biosystems), and standard amounts of MgCl2, PCR buffer, and dNTP. PCR was begun with a 5-min predenaturation cycle at 95°C, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 30 s. After being amplified, PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

**RT-PCR products (30 μl)** resulting from the nested primers were then digested with 20 U of AplI restriction enzyme (Roche Applied...
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RESULTS

Identification of the IGF-II Genomic Polymorphism in Barrett’s Esophagus. Genomic DNA from 43 Barrett’s esophagus biopsy samples were analyzed for the ApaI polymorphism in the IGF-II gene, which allows the detection of two IGF-II alleles. Two PCR-based methods were used to identify the IGF-II alleles. In one method, PCR amplification was performed using primers that flanked the IGF-II alleles. Two PCR-based methods were used to identify the IGF-II alleles. In one method, PCR amplification was performed using primers that flanked the IGF-II alleles. Two PCR-based methods were used to identify the IGF-II alleles. In one method, PCR amplification was performed using primers that flanked the IGF-II alleles. Two PCR-based methods were used to identify the

amplification of contaminating gDNA. (Analysis could not be performed on four patients with Barrett’s esophagus because the amount of remaining sample was insufficient.) Of the nine Barrett’s esophagus samples, five (56%) demonstrated GOAGE of IGF-II as determined by the finding of biallelic gene expression (Fig. 4). Of those five samples, four were from patients with long-segment Barrett’s esophagus and one sample exhibited low-grade dysplasia.

Gain of Allelic Gene Expression in Matched Normal Mucosal Samples. cDNA from matching normal duodenal and gastric biopsies from six of the nine patients with Barrett’s esophagus demonstrating GOAGE of IGF-II were analyzed to determine whether GOAGE of IGF-II was present in normal tissues. (Analysis could not be performed on 3 informative patients with Barrett’s esophagus because of insufficient tissue sam-

Fig. 1. Identification of the ApaI polymorphism of IGF-II by the conventional ApaI restriction enzyme digestion method in genomic DNA (gDNA) from metaplastic Barrett’s esophagus. Informative samples were those containing the ApaI polymorphism in either of the two IGF-II alleles. The polymorphism was identified by detecting three distinct bands on the gel after overnight digestion with ApaI. Representative samples 6B, 26B, 29B, 40B, 45B, and 46B demonstrate the 3 distinct bands detected in patients with the polymorphism and thus were informative for the IGF-II alleles. In samples 52B, 53B, and 59B, both IGF-II alleles contained the ApaI restriction enzyme site.

Fig. 2. Identification of the ApaI polymorphism in IGF-II using allele-specific PCR in gDNA from metaplastic Barrett’s esophagus. Representative samples 51B, 53B, and 54B demonstrate both allele A (allele with the polymorphism) and allele B (allele without the polymorphism) and thus are informative for IGF-II. Samples 52B, 55B, 57B, 58B, 59B, 60B, 61B, and 62B contain only one of the two alleles for IGF-II.
developed in which the sequences of the 3’ oligonucleotide ends are absolutely complementary to the DNA template: one primer to the intact Apal restriction enzyme site and the other to the Apal site containing the polymorphism (29). This technique has been shown to be superior to conventional Apal digestion for investigating IGF-II imprinting (29). In our study, we used both the conventional Apal restriction enzyme digestion method and the allegedly superior allele-specific PCR method to identify the Apal genomic polymorphism in DNA from our patients with Barrett’s esophagus. We found no significant difference in the percentage of patients informative for IGF-II determined using these two methods, and in a subset of patients, we found that both methods produced identical results. Overall, we found a frequency of informative cases of 30% among our patients with Barrett’s esophagus, which is slightly less than the previously reported frequency range of 34–57% in other tissues (26).

Allele-specific PCR does not entirely eliminate the potential error of amplifying gDNA that are contaminating the cDNA preparations. To minimize this error, we treated our total RNA with DNase and followed this with reverse transcription to generate cDNA that were then amplified using exon connection RT-PCR. In this method, cDNA were first PCR amplified using primers that span exons 8 and 9 (the exon connection) of the IGF-II gene, followed by PCR using primers within exon 9 alone (26). By using primers that span exon 8 and 9 in the first of these two PCRs, the amplification of gDNA, which retains intervening intronic sequences, is markedly reduced. Using this technique, we assessed biallelic gene expression of IGF-II in cDNA from metaplastic Barrett’s, normal gastric, and normal duodenal epithelia from nine informative patients with Barrett’s esophagus. We found GOAGE of IGF-II in five (56%) of nine samples of Barrett’s specialized intestinal metaplasia. To our knowledge, this study is the first to demonstrate IGF-II GOAGE in a metaplastic epithelium. The frequency of GOAGE of IGF-II has been reported to range from 25 to 100% in a variety of human tumors (9, 27). In the past few years, however, it has become clear that GOAGE is not limited solely to neoplastic tissue. Indeed, biallelic gene expression of IGF-II has been found in 12–17% of samples of colonic tissue from individuals without colonic neoplasia (6, 27). Furthermore, GOAGE of IGF-II has been found in peripheral blood lymphocytes from patients with a personal or familial history of colorectal neoplasia (4). This finding of biallelic IGF-II ex-

**Fig. 3.** Identification of the Apal polymorphism in IGF-II using allele-specific PCR in gDNA from metaplastic Barrett’s esophagus previously analyzed using the Apal restriction enzyme digestion method. Representative samples 29B and 45B demonstrate both allele A and allele B, indicating the presence of the Apal polymorphism and thus are informative for IGF-II. The same results were found on the basis of analysis using the Apal restriction enzyme digestion method shown in Fig. 1.

**Fig. 4.** Identification of gain of allelic gene expression (GOAGE) of IGF-II using exon connection PCR in cDNA from informative samples of metaplastic Barrett’s esophagus and normal gastric and duodenal samples. Representative Barrett’s samples 45B, 46B, 53B, and 54B demonstrate biallelic expression of IGF-II. Samples 29B and 51B retain monoallelic expression of IGF-II. Like the corresponding Barrett’s tissues, the normal gastric (G) and duodenal (D) samples from patient 51 demonstrated monoallelic IGF-II expression, whereas biallelic expression of IGF-II is detected in the duodenal and gastric samples from patients 53 and 54, respectively.

**DISCUSSION**

We found a gain in allelic gene expression for IGF-II in five (56%) of nine informative samples of Barrett’s specialized intestinal metaplasia. Genomic imprinting is a reversible epigenetic process involving differential DNA methylation between parental alleles (i.e., one allele is methylated and thereby silenced, whereas the other is not). This leads to differential expression of the maternal and paternal genes in somatic cells during development and in adult life. It has been established that the IGF-II gene is normally imprinted so that only the paternal allele is expressed (7). However, if this differential methylation is lost (i.e., when there is LOI), there is a gain in gene expression as a result of IGF-II being expressed by both alleles. GOAGE for the IGF-II gene has been documented in a number of human tumors, including squamous cell carcinoma of the esophagus (3, 14). Expression of IGF-I-R, the receptor through which IGF-II exerts its mitogenic effects, has been detected in metaplastic Barrett’s esophagus. IGF-I-R expression has been shown to increase as the degree of dysplasia in Barrett’s epithelium increases, a phenomenon suggesting a role for the IGF pathway in the neoplastic progression of Barrett’s esophagus (10). To our knowledge, our present report is the first to demonstrate GOAGE of IGF-II in metaplastic Barrett’s esophagus.

In most other studies of IGF-II GOAGE, genomic polymorphism identification and IGF-II allelic expression have been assessed on the basis of PCR or RT-PCR in combination with Apal restriction enzyme digestion. However, these methods can be flawed for various reasons, including partial digestion of the PCR products, the presence of digestion-resistant heteroduplexes in the PCR products, and contamination by gDNA (16, 18). These flaws might be responsible for the wide variations in the incidence of IGF-II GOAGE reported in human tumors (29). To overcome the problems introduced by Apal digestion, the technique of allele-specific PCR was developed in which the sequences of the 3’ oligonucleotide ends were assessed on the basis of PCR or RT-PCR in combination with Apal digestion, the technique of allele-specific PCR was developed in which the sequences of the 3’ oligonucleotide ends.
pression in apparently normal blood lymphocytes suggests that IGF-II GOAGE may be part of a systemic genetic process. The fact that GOAGE in lymphocytes did not correlate with environmental factors such as smoking, alcohol consumption, use of nonsteroidal anti-inflammatory agents, or nutrient ingestion suggests that IGF-II GOAGE may not be acquired in adulthood, but rather can occur early in life (4).

We found GOAGE for IGF-II in five (56%) of nine informative cases of Barrett’s specialized intestinal metaplasia. Our study is the first to demonstrate GOAGE for IGF-II in metaplastic Barrett’s epithelium. Although the nonneoplastic colonic epithelium surrounding colonic neoplasm has been shown to exhibit GOAGE for IGF-II, the investigators did not search for GOAGE in extracolonic tissues (6, 27). In our patients who had GOAGE for IGF-II in Barrett’s epithelium, we also found IGF-II GOAGE in biopsy specimens from the stomach and the duodenum. In contrast, the normal tissues from patients whose metaplastic epithelium retained monoallelic expression of IGF-II did not demonstrate GOAGE. These findings further support the hypothesis that GOAGE is a systemic genetic feature but do not provide insight into whether this feature is an inherited characteristic or an epigenetic alteration that occurs during early development (12).

In conclusion, we have shown that GOAGE for IGF-II occurs frequently in the metaplastic mucosa of patients with long- and short-segment Barrett’s esophagus. We have also shown that GOAGE for IGF-II occurs in normal gastric and duodenal epithelia from Barrett’s patients in whom the metaplastic epithelium demonstrates GOAGE, but not in those without GOAGE in Barrett’s esophagus. To our knowledge, this report is the first to describe GOAGE of IGF-II occurring in the metaplastic epithelium of patients with Barrett’s esophagus.

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REFERENCES