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Down-regulation of *Epidermal Growth Factor Receptor* by Selective Expansion of a 5′-End Regulatory Dinucleotide Repeat in Colon Cancer with Microsatellite Instability

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Abstract

Purpose—The *epidermal growth factor receptor* (*EGFR*) is overexpressed in several tumor types, and its expression is influenced by the length of a 5′-end microsatellite repeat $(CA)_n$: the longer the repeat, the lower the expression. Dinucleotide repeats accumulate insertion/deletion types of mutations in tumors with microsatellite instability. We designed this study to estimate the occurrence of these mutations in *EGFR*$(CA)_n$ and their relevance in carcinogenesis of microsatellite instability – positive colon and gastric tumors.

Experimental Design—We analyzed the frequency of *EGFR*$(CA)_n$ mutations *in vivo* in 55 colorectal and 14 gastric microsatellite instability – positive cancers, and *in vitro* in single-cell clone cultures of microsatellite instability – positive colon tumor cell line LS174. Single-cell clone cultures with different repeat lengths were analyzed by fluorescent-activated cell sorter for EGFR cell-surface expression. A correlation analysis was done between *EGFR*$(CA)_n$ mutations and mutations in *KRAS, BRAF*, and *p53*.

Results—Unlike single-cell clone cultures, which exhibited higher rate of deletions compared with insertions, most of *EGFR*$(CA)_n$ mutations in colon and gastric tumors were insertions. Longer *EGFR* $(CA)_n$ correlated with lower EGFR cell-surface expression in single-cell clone cultures. In colon cancers, the elongation of the repeat was associated negatively with mutations in *KRAS* and *BRAF*, but not in *p53*.

Conclusions—The *EGFR*$(CA)_n$ elongation observedin tumors cannot be explained by anintrinsic property of this repeat favoring insertions versus deletions. Instead, a selection for repeat elongation occurs in microsatellite instability – positive tumors, leading to *EGFR* down-regulation. These findings suggest that in microsatellite instability – positive tumors current therapies targeting EGFR overexpression may have either no effect or an opposite to the expected effect.

The *epidermal growth factor receptor* (*EGFR*), a member of the *erbB* gene family, encoding a tyrosine kinase receptor, regulates cell growth and survival by binding specific ligands such as epidermal growth factor, transforming growth factor α (TGF-α), and heparin-binding

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

epidermal growth factor (1,2). Over-expression of *EGFR*, which has been detected in a variety of cancer types, is believed to contribute to neoplastic transformation and is associated with a poor cancer prognosis (3,4). Overexpression of *EGFR* in glioblastomas can be explained by gene amplification (5), but amplification of *EGFR* in other types of cancer occurs only in a small percentage of tumors. Therefore, it is widely accepted that *EGFR* overexpression is regulated at the transcriptional level.

Transcription of *EGFR* starts at several initiation sites within a GC-rich promoter region (6). Two enhancer elements have been identified for *EGFR*: one upstream of the promoter and another in intron 1 (7). It has been also shown that the basal *EGFR* gene expression is influenced by the length of a microsatellite repeat sequence $(CA)_n$ located in the proximity of the intron 1 enhancer: the longer the repeat length, the lower the gene expression (8,9).

Microsatellites are unstable repetitive sequences and for this reason are not only "naturally" highly polymorphic in the human population but also may be abnormally mutated in tumors with compromised mismatch repair. Genetic or epigenetic inactivation of mismatch repair genes causes a mutator phenotype, which was discovered by the ubiquitous presence of slippage-induced insertion/deletion mutations in microsatellite sequences (10–12). When microsatellite instability takes place in a tumor precursor cell, it leads to the accumulation of many mutations, some of which occur in the coding regions of cancer genes, contributing to neoplastic growth (reviewed in ref. 13).

Translational Relevance

EGFR is overexpressed in different tumor types, and its overexpression correlates with malignant progression. Thus, we were intrigued by finding elongation of a microsatellite repeat at the 5′ untranslated region in gastrointestinal cancers with microsatellite instability because it had been reported that elongation reduced expression. Our study indicates that, indeed, this tendency for elongation led to down-regulation of the gene product. Therefore, the selection for *EGFR* down-regulation during tumorigenesis implies that the gene works as a tumor suppressor and not as an oncogene in gastric and colon cancer of the microsatellite mutator phenotype.

The immediate consequence of these findings has translational potential related to *EGFR*-targeted cancer therapies. Numerous cancer therapies, ranging from therapeutic and imaging antibodies to toxin-linked ligands for enhancement of targeting for gene therapy vectors, have used up-regulated *EGFR* as a tumor-specific target. Our findings predict that some of these therapies may have no effect or an opposite to the expected effect on microsatellite instability – positive colon tumors. It also remains to be determined whether this opposite effect of epidermal growth factor signaling in microsatellite instability – positive colon cancer compared with breast cancer, forinstance, is restricted to the mutator pathway or is extensive to other cancers.

In this study, we have analyzed somatic mutations in the *EGFR*$(CA)_n$ microsatellite in microsatellite instability–positive gastric and colon tumors. We have further studied the relationship between the mutations in the 5′*EGFR*$(CA)_n$ and 3′*EGFR* $(A)_n$ noncoding microsatellite sequences, and the mutations in the *KRAS*, *BRAF*, and *p53* cancer genes.

Materials and Methods

Tumor samples, cell lines, and microsatellite instability analysis

Co-lorectal and gastric tumors were obtained from the Cooperative Human Tissue Network (University of Alabama, Birmingham, AL). From a consecutive series of >500 unselected

colorectal normal-tumor matched pairs, we selected for EGFR repeat analysis the microsatellite instability–positive cases for which DNA was available ($n = 55$). Sixty-one MSS colorectal cancers were randomly selected for comparative purposes. From the 52 microsatellite instability – positive cases for which mutation data was available, some of the cases could be classified as HNPCC ($n = 5$) or familial cases ($n = 4$), some had no family history (i.e., sporadic, $n = 8$), and for the rest, no family history information was available ($n = 36$). Fourteen microsatellite instability and 53 MSS gastric cancers were also analyzed. Genomic DNA from frozen specimens was extracted with phenol-chloroform. The LS174T colon cancer cell line was obtained from the American Type Culture Collection and was grown in DMEM supplemented with 15% of fetal bovine serum (Tissue Culture Biologicals). Microsatellite instability status in primary tumors was analyzed by PCR, as described previously (8,14).

PCR analysis of the *EGFR* $(CA)_n$ length

The following primers were used for PCR amplification of the *EGFR* $(CA)_n$-containing region within the first intron of *EGFR*: 5′-GTTTGAAGAATTTGAGCCAACC-3′ and 5′-TTCTTCTGCACACTTGGCAC-3′. PCR was carried out using a mix of Taq (Perkin Elmer) and *Pfu* DNA polymerases (Stratagene; ratio, 1:0.01) in the presence of 0.2 mCi of [α-P^{32}] dCTP as follows: incubation at 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and one last incubation at 72°C for 10 min. PCR products were resolved on a 6% Sequencing gel (National Diagnostics) and subjected to autoradiography.

Isolation of the LS174T subclones with different numbers of CA repeats

Subclones with different $(CA)_n$ repeat numbers of LS174T cells were isolated as described (15). Cells were lysed in 100 μL of Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA; pH 7.4) supplemented with 150 μg/mL proteinase K and heated at 65°C for 1 h. The lysates were used for PCR amplification of the $(CA)_n$ region of *EGFR*. Two or three independent subclones with the same allele length were collected for further fluorescent-activated cell sorter analysis.

Frequency of insertion/deletion mutations in *EGFR* $(CA)_n$ in LS174T colon cancer cell line

To measure the frequency of mutations in *EGFR* $(CA)_n$, we followed the Luria and Delbruck approach (16). The LS174T subclone culture was used to start 48 independent single cell cultures, which were grown separately for 25 cell replications. Each independent culture was then subcloned to obtain 96 single-cell clones, which were used for PCR analysis of the $(CA)_n$ length. A change in the repeat length by one CA unit was considered a single mutational event. Hence, a $(CA)_{n+2}$ allele was scored as having undergone two insertions, and a $(CA)_{n-2}$ allele was scored as having two deletions. After 25 cell replications, the total number of insertions and deletions was calculated in every one of the 48 independent cultures, analyzing at least 30 subclones per culture. The total number of insertions and deletions was then divided by the number of subclones analyzed in that particular culture to obtain the frequency of elongation and shortening, respectively. These independently estimated frequencies were subsequently averaged to account for fluctuations in the mutational frequency among the different 48 cultures.

Analysis of EGFR cell surface expression in LS174T subclones

Cells from two or three LS174T independent subclones with the same allele length were harvested with a nonenzymatic cell dissociation reagent (Specialty Media) and used for fluorescent-activated cell sorter analysis. Cells (5×10^5) from each subclone were incubated with EGFR-specific monoclonal antibodies (mouse immunoglobulin G; clone EGFR.1 from BD Pharmingen) at 5 μg/μL for 30 min at 4°C (fluorescent-activated cell sorter buffer: 1% bovine serum albumin, 1 mmol/L Ca^{2+}, 1 mmol/L Mg^{2+}, 0.02% NaN_3 in PBS). Isotype-matched immunoglobulin G were used as negative control. Primary antibody incubation was

followed by washing with fluorescent-activated cell sorter buffer and incubating with FITC-labeled secondary antibodies at 5 μg/μL for 30 min at 4°C (BD Biosciences). Fluorescence was detected on a FACScan flow cytometer (BD Biosciences) using standard procedures. The experiment was repeated twice from different independent clone cultures.

KRAS, p53, and *BRAF* mutation analysis

KRAS mutations at codons 12 and 13 and *p53* mutations in exons 4 to 9 were analyzed by single-strand conformational polymorphism and sequencing, as described previously (18,19). *BRAF* mutations in exons 11 and 15 were determined by sequencing, as described (20).

Analysis of the polyA tract of the 3′ untranslated region (UTR) of *EGFR*

Specific primers were designed to amplify the polyA tract in the 3′ UTR of *EGFR* (forward, 5′-GAAACGCATCCAGCAAGAAT-3′; reverse, 5′-ACTCCAAGATCCCCAATCAA-3′). Reverse primer was labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase (Promega). PCRs were done in standard conditions (Roche Applied Science) as follows: a single incubation at 94°C for 3 min followed by 30 cycles at 94°C for 45s, 58°C for 45 s, and 72°C for 45 s, and one final incubation at 72°C for 10 min. PCR products were resolved on a 6% Sequencing gel (National Diagnostics) and autoradiographed. The allele size was assessed by direct DNA sequencing using an automated DNA Analyzer (Applied Biosystems).

Results

Elongation of *EGFR* $(CA)_n$ in microsatellite instability–positive gastric and colon tumors

We examined *EGFR* $(CA)_n$ for somatic insertion/deletion mutations in microsatellite instability–positive stomach and colon cancers. The $(CA)_n$ sequence was PCR amplified from pairs of tumor and matched normal tissue DNA and their length determined by PAGE (Fig. 1A). The most frequent $(CA)_n$ alleles in normal tissues contained 16, 18, and 20 CA units with frequencies 42%, 16%, and 17%, respectively (Fig. 1B). This was in accordance with previously published data (21). As for cancer tissues, 82% of the colon and 62% of the stomach exhibited mutations in at least one of the two $(CA)_n$ alleles.

Surprisingly, most of these mutations were insertions, resulting in elongation of the repeat (Fig. 1). Overall, 60 alleles with insertions versus 13 with deletions and 22 alleles with insertions versus 0 with deletions were detected in colon and gastric cancers, respectively (Table 1). The insertions ranged from 1 to 35 repeat units for colon and from 1 to 9 for gastric cancer. Length distribution of the *CA* alleles in tumor and normal tissues is shown in Fig. 1B. Eight alleles with repeats from 15 to 22 CA units were found in normal tissues of 69 gastrointestinal cancer patients, whereas 17 alleles with 14 to 53 CA repeats were found in the matched tumors. The allele with 16 CA repeats was the most prevalent in normal tissues (42.0%, 58 of 138). Its frequency was significantly lower in the tumor tissues (10.1%, 14 of 138; $P = 1.62 \times 10^{-9}$).

Expression of *EGFR* in microsatellite instability–positive and –negative colon tumors

EGFR expression was estimated by quantitative reverse transcription-PCR in 14 colon microsatellite instability and 38 MSS cancers for which RNA was available. Although there was a slight increase in the overall expression levels of *EGFR* mRNA in MSS tumors relative to matched normal tissues (108%), the average level of expression was about half (56%) in the microsatellite instability tumors ($P = 0.008$; data not shown).

Frequency of insertion and deletion mutations in *EGFR* $(CA)_n$ in vitro

To compare insertion and deletion mutation rates for the $(CA)_n$ repeat *in vitro*, we used the Luria and Delbruck approach (16). For this purpose, 48 single-cell clone subcultures of the

LS174T colon cancer cell line all with the same $(CA)_n$ allele sizes 20/28 were started. The subcultures were cultivated separately for 25 cell divisions, and the frequency of the CA repeat mutations were determined for each subculture. This was done by isolating 96 single-cell clones from each of the 48 independent cultures and analyzing their CA repeat length by PCR. A change in the repeat length by one CA unit was considered a single mutational event.

Average frequencies of deletions and insertions for the short $(CA)_{20}$ and the long $(CA)_{28}$ alleles were calculated over 48 independently grown cultures (Table 2) to accommodate for fluctuation of mutation frequency. Average frequencies of short and long allele mutations were used to compare their mutation rate. Short (1626) and long (1508) alleles were analyzed. The long allele had significantly higher rate of deletions versus insertions *in vitro* as measured by a higher average frequency of deletions versus insertions ($\chi^2 = 19.89$; $P = 0.001$). The short allele also had more deletions than insertions, although the difference was not statistically significant.

These data illustrate that the expansion of *EGFR* $(CA)_n$ observed in microsatellite instability–positive tumors cannot be explained by an intrinsic property of this repeat favoring insertions versus deletions. Instead, the expansion of the repeat observed in the tumors must be the result of selection in favor of insertions due to clonal advantage *in vivo*.

Modulation of *EGFR* expression by the number of CA units in *EGFR* $(CA)_n$

We examined *EGFR* cell surface expression by fluorescent-activated cell sorter analysis using monoclonal EGFR-specific antibodies. Binding to primary antibodies was visualized with the secondary FITC-labeled antibodies. The experiment was done with selected subclones of LS174T cell line, all sharing the same long allele of 28 CA units but different in the length of the short allele: 18, 19, 20, 21, 22, and 24 CA units.

Figure 2 shows that EGFR cell surface expression decreased almost 3-fold with the increase of the length of the short allele from 18 to 24 CA units. The expression level could be approximated by a formula $y = 139.41e^{-0.1844X}$, where X is the number of CA units and y is the relative EGFR cell surface expression. We used this trend line formula to calculate EGFR expression for allele sizes outside the 18 to 24 CA size range.

Our experimentally estimated relative levels of *EGFR* expression for different allele sizes agreed well with previously published *in vitro* transcription data for repeat sizes <20 CA units (8,9). This result illustrates that the modulation of *EGFR* expression occurs continuously throughout a wide range of repeat lengths in cells of different tissue origin.

Correlation between *EGFR* $(CA)_n$ repeat expansion and mutations in *KRAS, BRAF*, and *p53*

To explore the possibility that the expansions of the *EGFR* repeat could be related to the mutational status of *KRAS, BRAF*, and *p53*, we did correlation studies between the *EGFR* $(CA)_n$ length and the presence of mutations in these genes in microsatellite instability–positive colon cancers. We used the results of previous screening of these tumors for mutations in codons 12 and 13 of *KRAS* (exon 1), in exon 15 of *BRAF*, and in the coding region of *p53* from exons 4 to 9 (Table 3).

There was a negative association between the level of *EGFR* $(CA)_n$ expansion and mutations in either *KRAS* or *BRAF* (*t* test; $P = 0.01$). Tumors with mutations in either one of these two genes had less frequent insertions than the tumors without (Fig. 3, *top*). On the other hand, no correlation between mutations in *p53* and *EGFR* $(CA)_n$ repeat elongation was found (*t* test; $P = 0.74$).

Analysis of the polyA tract of the *EGFR* 3′ UTR

The 3′ UTR of *EGFR* harbors a polyadenine tract that is susceptible to mutation in microsatellite instability–positive tumors. Previous studies have shown that mutations in the 3′ UTR could modulate gene expression (22,23). We determined the length of this *polyA* tract in 40 microsatellite instability–positive colon tumors and corresponding normal tissues. *EGFR* $(A)_n$ is polymorphic in the normal samples: the most common alleles contain 13 or 14 adenines. As expected, most tumors (92.5%) showed a shortening in this sequence (12). There was no correlation between the number of deletions in the 3′-end *poly*$(A)_n$ and the number of CA insertions in the $(CA)_n$ of the first intron of *EGFR*. No correlation between the deletions in the *poly*$(A)_n$ of *EGFR* and mutations in the *KRAS* and *BRAF* oncogenes was found (Fig. 3, *bottom*).

We also examined the length of *EGFR* $(A)_n$ in the LS174T subclones with different numbers of CA repeats and found that all of the subclones used in the fluorescent-activated cell sorter analysis were haploidentical. Therefore, the difference in *EGFR* expression observed in these clones was due solely to the changes in the number of $(CA)_n$ units in the 5′ end of the gene and not to alterations in the 3′-end noncoding repeat.

Discussion

EGFR is overexpressed in several tumors, and its over-expression correlates with malignant progression (24). Thus, we were intrigued by finding elongation of a microsatellite repeat at the 5′ UTR in gastrointestinal cancers with micro-satellite instability because it had been reported that elongation reduced expression (8,9). Our study indicates that indeed this tendency for elongation that leads to down-regulation of the gene product is not due to an intrinsic tendency of the DNA sequence. Therefore, the selection for *EGFR* down-regulation during tumorigenesis implies that, in gastric and colon cancer of the microsatellite mutator phenotype, the gene works as a tumor suppressor and not as an oncogene. These observations may not be contradictory to each other, considering the differences between tumorigenic pathways of microsatellite instability–positive and microsatellite instability–negative cancers, and the pleiotropy of the biological responses to *EGFR* activation.

The research history of TGFs is an excellent example of divergent growth roles of proteins in different cell contexts. TGFs were initially detected and isolated by their transforming activities in normal anchorage-dependent cells (25,26). It was only later that it became apparent that the common effects of these TGFs were of a negative nature. Thus, TGF-β plays a preferentially repressor function, despite its misleading name (27).

Although overexpression of *EGFR* was detected in several tumor types, particularly brain and breast, there is also a report that the EGFR level is lower in carcinomatous than in normal colorectal tissues (28). Because one of the EGFR ligands is TGF-α, down-regulation of *EGFR* in microsatellite instability–positive tumors may have an effect similar, additive or synergistic, to the mutational inactivation of the TGF-β receptor type II gene, which is a major event in the tumorigenesis of microsatellite instability – positive tumors (29).

In contrast to the accepted positive growth role of EGFR in breast and brain tumorigenesis, ligand stimulation of cells that overproduce EGFR may result in apoptosis, and apoptosis is a predictable outcome of *EGFR* overexpression in a variety of cell types (30,31). To explain this apparent paradox, the latter authors proposed that during the clonal evolution of a tumor, low levels of receptor activation may enhance survival and indirectly promote mitogenesis and confer growth advantage to cells.

It has been shown that the induction of apoptosis by exposure of *EGFR*-overexpressing cells to epidermal growth factor is enhanced by inhibition of RAS signaling. The Raf–MAP/ERK kinase–extracellular signal-regulated kinase (mitogen-activated protein kinase) signal transduction is an important mediator in cell growth, proliferation, and survival. *BRAF* is activated by oncogenic *RAS*, leading to cooperative effects in cells responding to growth factor signals. The cells that expressed a dominant negative RAS/BRAF mutant exhibited a significant enhancement of EGFR-induced apoptosis, suggesting that *RAS* activation is a key survival signal generated by EGFR (31). Microsatellite instability–positive tumors are characterized by a paradoxically low incidence of *RAS* mutations (6,32). Thus, microsatellite instability–positive tumor cells that carry normal RAS and BRAF proteins may be under selective pressure to down-regulate *EGFR* expression to escape apoptosis. These circumstances might cause a negative association between the presence of *RAS/BRAF* mutations and the *EGFR* $(CA)_n$ repeat expansion.

We failed to find any *EGFR* $(CA)_n$ mutations in 53 stomach and 61 colon microsatellite instability–negative tumors. The negative result is not surprising because the rate of insertion/deletion mutations in microsatellite instability – positive tumors is three orders of magnitude higher than in tumors without microsatellite instability. In this context, expression of the *EGFR* gene *in vivo*, analyzed by immunohistochemistry using a specific antibody, showed that *EGFR* expression was inhibited in some of the microsatellite instability–positive colon cancers analyzed, whereas staining was generally strong in microsatellite instability–negative tumors (data not shown).

The complex and pleiotropic role of the EGFR signaling system may dictate an alternative use of the EGFR signaling pathway during tumorigenesis, depending on the cancer organ site and the mechanism of cancer development. A microsatellite instability–positive tumor precursor cell produces many mutations without yet manifesting a neoplastic phenotype. Inactivation of DNA mismatch repair and the resulting unrepaired genomic errors may serve as apoptotic signals. The elongation of *EGFR* $(CA)_n$ occurs with high frequency because of the intrinsic instability of microsatellite sequences. The immediate consequential down-regulation of the gene expression may hamper the apoptotic response of the cell and help its survival until more powerful mechanisms of apoptosis inhibition are triggered, for example, an inactivating frameshift mutation in the mononucleotide stretch of the *BAX* gene (33).

If mutational inactivation of the apoptotic machinery occurs, the down-regulation of *EGFR* may no longer be required and the elongated *EGFR* $(CA)_n$ sequence could shorten to a stable size during further growth of the tumor or tumor precursor cell. However, it is possible that the partial inhibition of apoptosis by *EGFR* down-regulation early in tumor development may never lose its selective advantage. In contrast to microsatellite instability–negative tumors, for which the two-hit tumor suppressor gene inactivation model (34) applies so well, microsatellite instability–positive tumors are characterized by the presence of a plethora of monoallelic mutations in cancer-related genes (8,35,36). Such monoallelic mutations can cause haploinsufficiency and partial inactivation of gene function, which, when occurring simultaneously in many genes of the same oncogenic signaling networks, may bring down the normal homeostasis in cell growth or survival.

In microsatellite instability–positive cells, such mechanism would lead to a cascade of inactivation of tumor suppressor function analogous to the cascade increase of mutator phenotype, which we also observed for these tumors (15,37,38). In this case, each of these small effects, such as down-regulation of *EGFR* gene expression, heterozygous frameshift mutations in *BAX*, and other apoptotic genes (33,35), can presumably cause altogether a significant inhibition of apoptosis and may remain under a selective pressure during tumor development and progression.

Thus, although EGFR was one of the first discovered factors implicated in the process of malignant transformation, its complex role in tumorigenesis may be far from having been completely understood. As an illustrative example, a recent report described *EGFR* to be frequently hypermethylated and silenced in several cancers, including breast, lung, and head and neck carcinomas (39).

The immediate consequence of these findings has translational potential related to *EGFR*-targeted cancer therapies. Numerous cancer therapies, ranging from therapeutic and imaging antibodies to toxin-linked ligands for enhancement of targeting for gene therapy vectors, have used up-regulated *EGFR* as a tumor-specific target (reviewed in ref. 40). Our findings predict that some of these therapies may have no effect or an opposite to the expected effect in microsatellite instability–positive gastrointestinal tumors.

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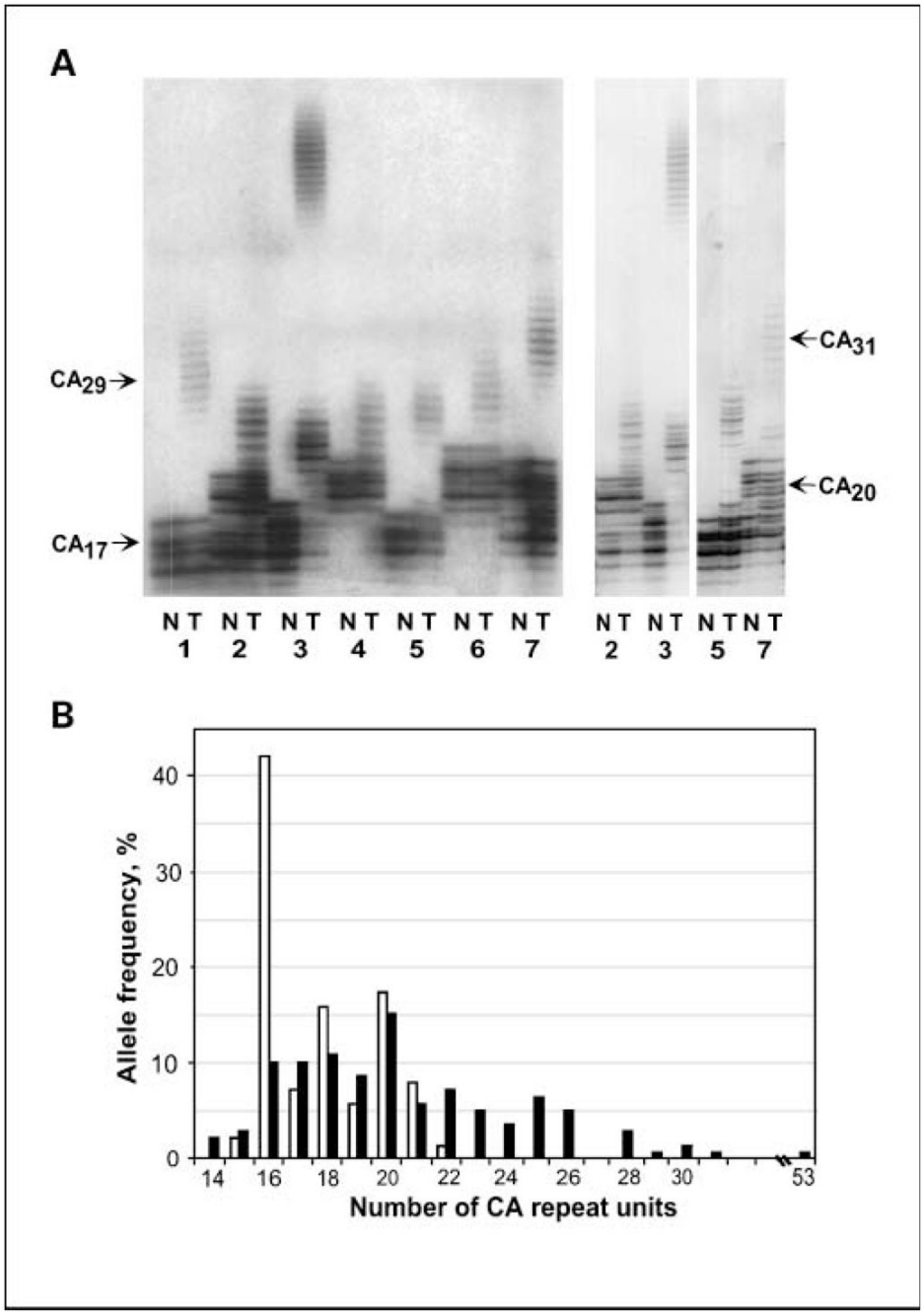

Fig. 1.

EGFR $(CA)_n$ mutations in microsatellite instability – positive tumors. *A*, electrophoregrams of $(CA)_n$-containing PCR fragments amplified from seven (1–7) microsatellite instability – positive colon tumor and matched normal tissues. Arrows, positions of bands that correspond to alleles with 17, 20, 29, and 31CA units. Right, a lower exposure of some of the samples at left from an independent experiment. *B*, distributionof *EGFR* allele length in microsatellite instability – positive gastrointestinal (52 colorectal and 14 gastric) tumors and matchednormal tissues. Openbars, normal tissue; closedbars, tumor tissue.

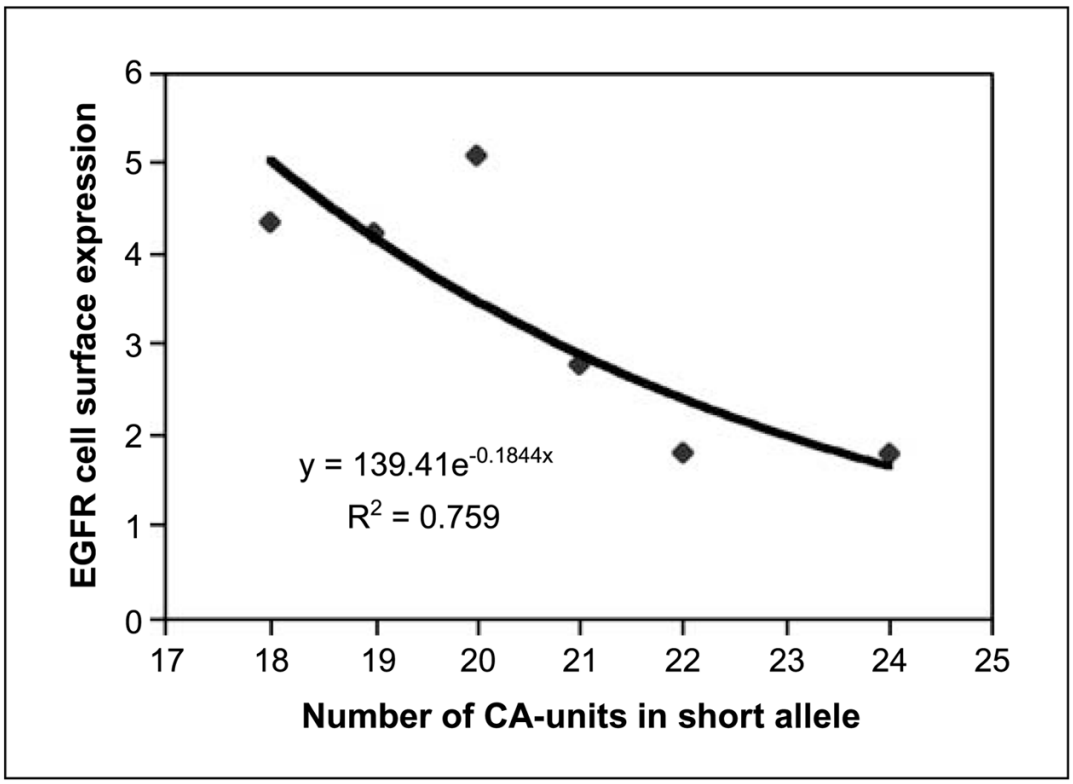

Fig. 2.

EGFR cell-surface expression in LS174T subclones with alleles of different $(CA)_n$ lengths. Each dot on the graph, the average value of the expression of at least two independent clones with the same allele length from two independent repeated experiments. The trend line was automatically drawn by Microsoft Excel software using $y = \alpha \times \exp(\beta \times X)$ formula for approximation of the experimental results. The exact formula is shown inside the chart area.

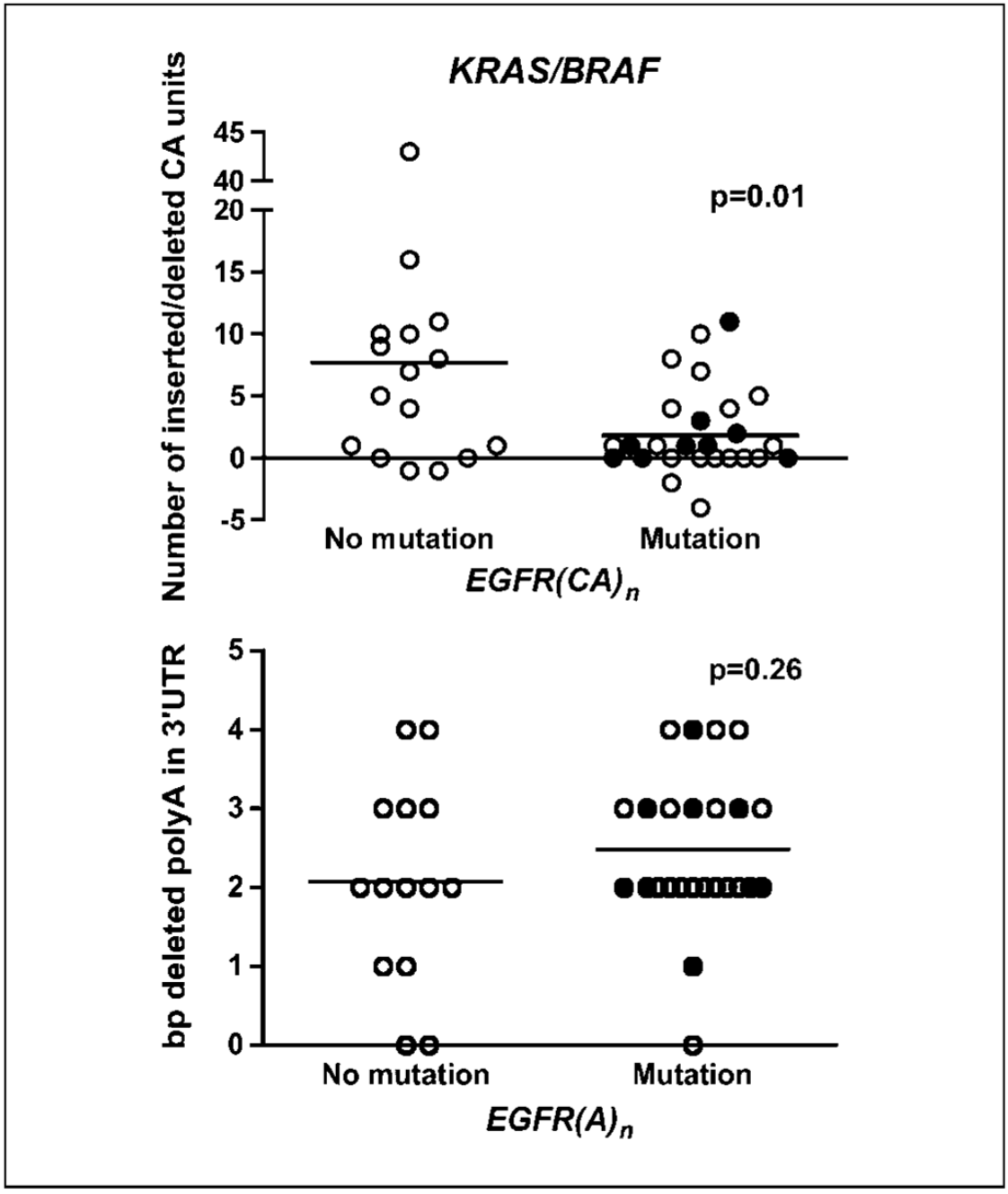

Fig. 3.

Association analysis between insertions in *EGFR* $(CA)_n$ (*top*) and deletions in *EGFR* 3′ UTR $(A)_n$ (*bottom*), and mutations in *KRAS* (*solid dots*) and *BRAF* (*empty dots*). Similar trend was observed when analyzing separately microsatellite instability tumors with mutations in K-ras ($P = 0.13$; $n = X$) or in B-raf ($P = 0.03$; $n = X$) versus microsatellite instability tumors with no mutations in these genes. No cases were found with concomitant K-ras and B-raf mutations.

Table 1

Frequency of insertion and deletion types of mutations in *EGFR* $(CA)_n$ repeat in tumor tissues of gastrointestinal cancer patients

Mutations	Insertions	Deletions	No change
Colon cancer	54.6% (60/110)	11.8%(13/110)	33.6% (37/110)
Stomach cancer	78.6% (22/28)	0% (0/28)	21.4% (6/28)
Total	59.4% (82/138)	9.4% (13/138)	31.2% (43/138)

Table 2

Frequency of deletions and insertions in $(CA)_{20}$ and $(CA)_{28}$ alleles of *EGFR in vitro* as determined by Luria and Delbrück approach

Repeat	Frequency of shortening	Frequency of elongation
$(CA)_{20}$	0.147 ± 0.020	0.144 ± 0.018
$(CA)_{28}$	0.313 ± 0.025	0.193 ± 0.021

Table 3

EGFR $(CA)_n$, *KRAS*, *BRAF*, and *p53* genotypes for 52 microsatellite instability–positive colorectal cancers

Case no.	*EGFR*$(CA)_n$[†]	*EGFR*$(CA)_n$[‡]	*KRAS*[§]	*BRAF*[‖]	*p53*[¶]
1	+3/+6	+9	wt	wt	wt
2	+1/−1	0	wt	mut	wt
3	0/+12	+12[*]	wt	mut	Ala159Thr
4	0/+6	+6[*]	wt	wt	wt
5	+1/+3	+4	wt	mut	wt
6	0/0	0	mut	wt	Arg283Cys
7	0/+2	+2[*]	wt	wt	Glu326Gly
8	0/−1	−1	wt	wt	wt
9	+4/+1	+5	wt	mut	wt
10	0/+1	+1	mut	wt	Met246Val
11	−2/−2	−4	wt	mut	wt
12	+2/+9	+11	wt	wt	wt
13	+1/+2	+3	mut	wt	wt
14	0/+5	+5[*]	wt	mut	wt
15	+4/+6	+10	wt	wt	wt
16	0/+8	+8[*]	mut	wt	wt
17	+7/+9	+16	wt	wt	mut
18	0/+1	+1	wt	mut	wt
19	0/0	0	wt	wt	wt
20	0/+1	+1	wt	wt	Val73Met
21	+2/+5	+7	wt	mut	wt
22	+4/+4	+8	wt	mut	wt
23	0/+5	+5	wt	wt	wt
24	−2/+2	0	wt	mut	Arg175Cys
25	−1/+1	0	wt	mut	His168Arg
26	−1/0	−1	wt	wt	wt
27	−2/+3	+1	wt	wt	wt
28	−3/0	−3[*]	wt	mut	wt

Case no.	*EGFR*(CA)$_n$[†]	*EGFR*(CA)$_n$[‡]	*KRAS*[§]	*BRAF*[‖]	*p53*[¶]
29	+1/+10	+11	mut	wt	wt
30	−2/+3	+1	wt	mut	wt
31	−2/0	−2[*]	wt	mut	Thr155Asp
32	0/0	0	wt	wt	wt
33	0/0	0	mut	wt	wt
34	−2/0	−2	wt	mut	wt
35	0/+3	+3[*]	wt	wt	Gly245Asp
36	+1/+1	+2	mut	wt	wt
37	0/0	0	wt	mut	wt
38	+1/0	+1	mut	wt	wt
39	0/+1	+1	mut	wt	wt
40	+4/+6	+10	wt	mut	Arg181Cys
41	+8/+35	+43	wt	wt	wt
42	0/0	0	mut	wt	wt
43	0/0	0	wt	mut	wt
44	+1/+3	+4	wt	wt	wt
45	0/0	0	wt	mut	wt
46	0/+1	+1	wt	mut	wt
47	+1/−1	0	wt	mut	wt
48	+6/+4	+10	wt	wt	Gly154Ser
49	+3/+4	+7	wt	wt	wt
50	+1/+3	+4	wt	mut	wt
51	+2/+6	+8	wt	wt	wt
52	0/0	0	wt	mut	wt

Abbreviations: wt, wild type; mut, mutations.

[*]Asterisks denote noninformative cases in regards to whether one or two alleles underwent insertion/deletions. These 8 samples were excluded from the correlation study (Fig. 3). The result was not affected if the values of these cases were included. The interpretation of these cases was difficult due to the length similarity of constitutional and/or mutated alleles, together with the presence of significant contamination by normal tissue in the tumor tissue samples.

[†]Number of added (+) or deleted (−) CA repeat units in the two alleles (shorter/longer) in the tumor compared with the constitutional alleles.

[‡]Accumulative value in the number of mutated (CA) repeat units from the two EGFR alleles.

[§] *KRAS* mutations were Gly12Asp (cases 6, 10, 29, and 38), Gly12Ala (case 16), and Gly13Asp (cases 13, 33, 36, 39 and 613).

[||] *BRAF* mutations were exclusively the Val600Glu at exon 15. No mutations were identified in exon 11. Two mutations at exon 11 were identified among 72 microsatellite instability–negative colon cancers.

[¶] *p53* missense mutations. Case 7 has an additional silent nucleotide substitution in codon Ser96; the mutation in case 17 was only characterized by single-strand conformational polymorphism analysis.

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