

ras MUTATION AND EXPRESSION OF THE *ras*-REGULATED GENES OSTEOPONTIN AND CATHEPSIN L IN HUMAN ESOPHAGEAL CANCER

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As part of our ongoing studies to characterize molecular alterations in a well-defined series of surgically resected esophageal cancers, we examined the expression of 2 *ras*-regulated genes, whose products (osteopontin and cathepsin L) previously were shown to be associated with tumor invasion and metastasis. RNA was extracted from primary esophageal tumors (adenocarcinomas, 19; squamous-cell carcinomas, 6) and matched histologically normal esophageal mucosa from the distant resection margin. Northern analysis was used to quantitate RNA, relative to an 18S rRNA control, and immunohistochemistry to assess the tissue distribution of osteopontin. In addition, H-, K- and N-*ras* mutations were studied in the same tissues using PCR and hybridization with allele (mutant)-specific oligonucleotide probes. We demonstrated a K-*ras* mutation (codon 12, GTT) in one esophageal adenocarcinoma. The *ras*-regulated gene osteopontin was over-expressed in 100% of squamous-cell carcinomas and in 58% of adenocarcinomas relative to matched normal esophageal mucosa. Patterns of immunoreactivity for osteopontin protein also varied between squamous-cell carcinomas (tumor cell staining) and adenocarcinomas (predominantly tumor-infiltrating macrophages). Expression of cathepsin L also varied with esophageal tumor histology, with over-expression in 58% of primary esophageal adenocarcinomas and 33% of squamous-cell cancers. *Int. J. Cancer* 72:739–745, 1997.

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Carcinoma of the esophagus is one of the most frequent malignancies worldwide, with marked geographic variations in incidence. Although esophageal tumors are relatively uncommon in North America, epidemiologic studies have reported a marked increase in incidence for adenocarcinomas of the lower esophagus. Except for an association with Barrett's esophagus, a pre-malignant condition believed to result from gastro-esophageal reflux, little is known about the pathogenesis of primary esophageal adenocarcinomas. Studies of well-established risk factors, including alcohol consumption, smoking and diet, have shown inconsistent or reduced associations for primary esophageal adenocarcinomas compared with squamous-cell carcinoma. Further insight into the development and progression of esophageal cancer is anticipated from an improved understanding of its tumor biology and analysis of interactions between environmental factors and molecular genetic alterations in esophageal tumors and pre-malignant tissues.

The *p53* tumor-suppressor gene appears to have a central role in human neoplasia, and following initial reports of mutations in esophageal squamous-cell carcinomas (Hollstein *et al.*, 1990) and Barrett's adenocarcinomas (Casson *et al.*, 1991), considerable attention has been focused on this gene in esophageal tumorigenesis (for review, see Montesano *et al.*, 1996). However, it is clear that not all esophageal tumors have *p53* alterations, and it is therefore likely that other genes are involved in esophageal cancer development and progression. The *ras* family of genes initially appeared promising as these oncogenes are mutated frequently in human gastro-intestinal and upper aerodigestive tract (*i.e.*, oropharyngeal and lung) malignancies and in experimental rat esophageal cancer models (Wang *et al.*, 1990; Lozano *et al.*, 1994). Whereas

amplification of the K-*ras* oncogene (Galiana *et al.*, 1995), over-expression of H-*ras* RNA (Sorsdahl *et al.*, 1994) and p21 protein (Ruol *et al.*, 1990) have been reported, no *ras* mutations have been detected in human esophageal cancers to date. Interestingly, H- and K-*ras* mutations were found in a small number of highly tumorigenic esophageal cell lines, whereas the primary esophageal squamous-cell carcinoma from which the cell line was derived had no *ras* mutation (Galiana *et al.*, 1993). Furthermore, transfection of an activated H-*ras* gene into a non-tumorigenic esophageal cell line resulted in tumorigenicity *in vivo*, suggesting that *ras* indeed may have a role at some stage of multistep esophageal tumorigenesis. It is also possible that *ras*-induced tumorigenicity is due to the induction (or repression) of genes whose expression is regulated by *ras*, as well as by other signal pathways.

Osteopontin, a calcium-binding phosphoprotein, has been identified as a tumor-associated protein in various tumor cell lines in culture (for review, see Denhardt and Guo, 1993). Levels of osteopontin were increased in malignant, *ras*-transformed fibroblasts (Chambers *et al.*, 1992a), where its expression is regulated transcriptionally by *ras*-responsive elements in the osteopontin promoter (Guo *et al.*, 1995). Anti-sense down-regulation of osteopontin RNA has been shown to result in reduced malignancy (Behrend *et al.*, 1994), supporting the idea that osteopontin can contribute functionally to neoplasia. While osteopontin is known to be produced by many tumor cell lines in culture, little is known about its expression in human tumors, though we have reported osteopontin over-expression in carcinoma of the lung (Chambers *et al.*, 1996). An additional *ras*-induced gene is major excreted protein (MEP), the precursor of the cysteine proteinase cathepsin L (Denhardt *et al.*, 1986). Cathepsin L is elevated in many transformed cells, and increased levels have been associated with tumor invasion and metastasis (Chambers *et al.*, 1992b), 2 characteristic features of esophageal cancer thought to contribute to its dismal prognosis. To date, there has been little study of the molecular genetic events associated with the metastatic potential of esophageal carcinoma and, in particular, of *ras*-mediated changes in gene expression that may contribute to metastasis.

As part of our ongoing studies to characterize molecular alterations in a well-defined series of surgically resected esophageal cancers (predominantly adenocarcinomas), we examined the expression of 2 *ras*-regulated genes, osteopontin and cathepsin L,

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whose RNA products were quantitated using Northern analysis. Immunohistochemistry also was used to assess the tissue distribution of osteopontin. In addition, H-, K- and N-*ras* mutations were studied using PCR and hybridization with allele (mutant)-specific oligonucleotide probes. Anticipating a low frequency of *ras* mutations, we also studied *p53* alterations in the same esophageal tissues using PCR-based molecular assays, Northern analysis and immunohistochemistry. Molecular alterations were correlated with clinical and histo-pathologic features of these tumors.

MATERIAL AND METHODS

Patients and tumors

The study population comprised a sequentially accrued case series of 25 patients (21 male, 4 female) from a low-incidence region (southern Ontario, Canada) who underwent a curative resection of a histologically proven esophageal carcinoma at Victoria Hospital (a university teaching hospital and tertiary referral center), London, Ontario, Canada. No patient received pre-operative chemotherapy or radiotherapy.

Resected esophageal tissues were examined pathologically and staged according to the UICC TNM classification. Strict clinicopathologic criteria were used to define a primary esophageal (vs. gastric cardia) adenocarcinoma (Casson *et al.*, 1991), as follows: the presence of an associated Barrett's epithelium (columnar epithelium-lined esophagus extending greater than 3 cm above the anatomic esophagogastric junction or the presence of specialized "intestinal-type" epithelium), greater than 75% of the tumor mass involving the tubular esophagus, direct invasion of peri-esophageal tissues, minimal gastric involvement and clinical symptoms of esophageal obstruction (dysphagia). Tissues were placed in liquid nitrogen as soon as possible after resection and stored in the esophageal tumor bank at -80°C for molecular analysis. Tumor histology was confirmed by frozen section analysis at the time of processing, and all banked tumor tissue comprised $>80\%$ malignant cells, with minimal necrosis. For each tumor, matched histologically normal esophageal mucosa, from the distant resection margin, was available as an internal control. Resected esophageal tissues (tumor and matched normal mucosa) were formalin-fixed, and paraffin-embedded for routine histology, using hematoxylin and eosin, and for immunohistochemical analysis.

Use of resected esophageal tissues was approved by the Review Board for health sciences research at the University of Western Ontario and by the Department of Pathology, Victoria Hospital.

RNA isolation and Northern blot analysis

Tissues (tumor and matched normal) were homogenized mechanically (Polytron PT 1200; Brinkmann, Mississauga, Canada) and RNA extracted using TRIzol Reagent (200 mg tissue/2 ml TRIzol; Life Technologies, Burlington, Canada), according to the protocol supplied by the manufacturer. RNA (10 $\mu\text{g}/\text{lane}$) was run on a 1.1% agarose gel with 6.8% formaldehyde and capillary-transferred to GeneScreen Plus filters (DuPont, Mississauga, Canada). Blots were probed with denatured, oligolabeled [^{32}P]-dCTP cDNA probes, using an oligolabeling kit (Pharmacia, Baie d'Urfé, Canada) according to the procedure provided by the manufacturer and as reported (Chambers *et al.*, 1992a,b, 1996; Sorsdahl *et al.*, 1994). RNA levels were quantified by densitometry (Phosphorimager SI; Molecular Dynamics, Sunnyvale, CA) and calculated as levels relative to the 18S control for each lane.

The probes used in these studies included a human osteopontin cDNA probe (Young *et al.*, 1990); a cathepsin L probe, which was the 1.19-kb cassette of plasmid pSP65-MEPA (Denhardt *et al.*, 1986); and a *p53* probe, which was a 1.2-kb human *p53* cDNA (Oncor, Gaithersburg, MD). The control probe was an 18S rRNA

(p100D9; a gift from Dr. D. Denhardt, Rutgers University, Piscataway, NJ). All assays were repeated at least 3 times.

Immunohistochemical analysis of osteopontin

Immunohistochemistry was used to study osteopontin in fixed tissues, as reported (Chambers *et al.*, 1996). The monoclonal antibody (MAb) 53, raised against recombinant human osteopontin (Bautista *et al.*, 1994), which recognizes intact (not thrombin-cleaved) native human osteopontin, was used at 1:750 dilution. Reagent and tissue controls were run in parallel, including blocking with recombinant osteopontin protein to ensure specificity of osteopontin detection. Osteopontin levels were expressed in each tissue section using a semi-quantitative scoring system. Briefly, this scored the percentage of positive staining cells (none = 0, $<1\%$ = 1, 1–10% = 2, 11–33% = 3, 34–66% = 4, $>67\%$ = 5), as well as the intensity of staining (none = 0, weak = 1, intermediate = 2, strong = 3). The proportion and intensity scores were added to give a final score (0, 2–8). In addition, the intensity of osteopontin staining in tumor-infiltrating macrophages was evaluated subjectively (none = 0, occasional or focal staining = 1+, weak = 2+, intermediate = 3+, strong = 4+). Tissue sections were evaluated independently by 2 investigators, one of whom was a consultant pathologist. Discordant cases were discussed at the double-headed microscope until consensus was reached.

DNA isolation and ras mutation analysis

Genomic DNA was extracted from both esophageal tumor and matched normal tissues according to standard methods of proteinase K/SDS digestion, phenol-chloroform extraction and ethanol precipitation. H-, K- and N-*ras* mutations in genomic DNA extracted from esophageal tissues were identified using PCR followed by hybridization with allele (mutant)-specific oligonucleotide probes. The PCR amplification primers (amplimers) for exons 1 and 2 of H-, K- and N-*ras* were purchased from Clontech (Paolo Alto, CA). A small portion of the PCR product was electrophoresed in a 12% polyacrylamide gel in $1\times$ TBE buffer at 200 V. The remaining PCR products were dot-blotted onto a Hybond-N nylon filter membrane (Amersham, Oakville, Canada) using a blotting manifold (GIBCO-BRL, Grand Island, NY); 4 μl of each amplified product were dot-blotted onto the filter, and 6 replicate dot-blot membranes were prepared for each set of products. The membranes were then cross-linked using a UV Stratilinker 1800 (Stratagene, La Jolla, CA).

The oligonucleotide probes for the wild-type and mutant alleles of codons 12, 13 and 61 of H-, K- and N-*ras* were those in the MUTA-LYZER kit (Clontech). Each codon-specific kit contains a panel of either 7 or 8 individual oligonucleotide probes that correspond to the wild-type and all pertinent mutations of the *ras* oncogene. Probes were terminally labeled with [γ - ^{32}P]ATP (6,000 Ci/mol) using T4 polynucleotide kinase. Filter membranes were pre-hybridized for 1–2 hr in a solution containing $5\times$ SSPE ($1\times$ contains 0.15 mol/l NaCl; 0.01 mol/l $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$; 0.001 mol/l $\text{Na}_2\text{-EDTA}$), $5\times$ Denhardt's solution ($1\times$ contains 0.2 gm/l polyvinylpyrrolidone; 0.2 gm/l BSA; 0.2 gm/l Ficoll 400), 0.5% SDS and 100 mM sodium pyrophosphate, at pH 7.5. Hybridization was carried out overnight at 55°C . The membrane was then washed once with $6\times$ SSC and 3 times in 3 M tetramethylammonium chloride (TMAC). Membranes were exposed to Kodak XAR-5 film at -70°C for 3 hr.

ras mutations were identified using 2-step screening hybridization. At the first screening, the mutant allele probes for each indexed codon were pooled into 2 groups of probe mixture. This allowed us to reduce the number of hybridization reactions that we had to carry out to evaluate all 57 potential mutations per sample. A positive control with the wild-type oligonucleotide probe was always carried out in parallel. If a mutation was detected, the dot-blots were stripped in an aqueous solution containing 0.1% SDS and $0.1\times$ SSC for 30 min at 75°C , then re-hybridized to

individual single allele mutant probes. However, since we knew in which group of pooled mutant probes the positive signal was found, this second-round hybridization was restricted to the mutant probes belonging to the mixture group. The positive control using the corresponding wild-type probe was again performed in parallel. The result of this second-round hybridization served 2 purposes: (i) to confirm the presence of the mutant gene and (ii) to localize the site of the point mutation.

Positive *ras* mutations and randomly selected DNA samples with wild-type *ras* were confirmed by DNA sequencing. PCR amplification was repeated using the same amplification primers and the product electrophoretically separated. The desired band was isolated, then blunt-end-ligated into the pGEM-T vector using the pGEMT-T Easy Vector System (Promega, Madison, WI). The DNA ligation reaction product was used to transform DH5 α -competent cells (GIBCO-BRL). Ten bacterial colonies were selected, and plasmid DNA was isolated using the mini-prep method. These were screened for the presence of mutant *ras* using the same dot-blot procedure as described for genomic DNA. Two micrograms of plasmid DNA with mutant *ras* were used to perform sequencing with the T7 Sequencing Kit (Pharmacia), using the original K-*ras* 12/13 5' or 3' primers and [α^{35} S]-dATP as label. Random samples with wild-type *ras* also were confirmed by sequence analysis.

p53 alterations

p53 mutations were detected using PCR/single-strand conformation polymorphism (SSCP) analysis of exons 4–10, as originally described (Casson *et al.*, 1991), with modification in primer location and electrophoresis conditions (Ozcelik *et al.*, 1995). All samples were re-amplified from the original genomic DNA, followed by a second PCR/SSCP analysis. Direct DNA sequencing (forward and reverse) was used to confirm the site and nature of the mutation.

A modified indirect immunoperoxidase technique was used to study the distribution of p53 protein in formalin-fixed, paraffin-embedded 4- μ m tissue sections, as reported previously (Casson *et al.*, 1995). Briefly, 2 primary anti-p53 antibodies (mutant and wild-type) were used in separate assays: the murine DO7 MAb and the rabbit polyclonal CM1 at 1:25 and 1:1,000 dilutions, respectively (Novacastra, Newcastle upon Tyne, UK). Controls were run in parallel with test sections and included known positive and negative staining tissues (tissue controls) and test sections processed without the primary antibody (reagent controls) and with matched histologically normal esophageal mucosa. To overcome

potential limitations of evaluating heterogenous tissues with variable immunoreactivity, both the fraction of positive staining cell nuclei and the average staining intensity were assessed (Allred *et al.*, 1993).

RESULTS

Tumor pathology

Esophageal tumor histology was squamous-cell carcinoma (24%, 6/25) or adenocarcinoma (76%, 19/25) with poor (64%, 16/25), moderately (24%, 6/25) or well- (12%, 3/25) differentiated tumors. Post-resection tumor stages (UICC, pTNM) were IIA (n = 7), IIB (n = 1), III (n = 15) and IV (n = 2).

Osteopontin RNA levels in esophageal tumors

Osteopontin expression in esophageal tumor tissues and matched histologically normal esophageal mucosa was assessed by Northern blotting and quantitative densitometry, relative to 18S rRNA levels to control for RNA loading (Fig. 1). Osteopontin RNA levels were generally low in all normal tissues. In contrast, osteopontin RNA was over-expressed (greater than 1.5 \times , relative to matched normals) in all squamous-cell carcinomas (100%, 6/6; range, 1.7 \times –4.5 \times) and in 58% of adenocarcinomas (11/19; range 1.9 \times –8.7 \times). These data are summarized in Table I.

Immunohistochemical detection of OPN protein

Osteopontin protein levels in corresponding sections of esophageal tumor and matched normal tissues were assessed using the anti-osteopontin mAB53 (Table I). Normal esophageal tissues were uniformly negative for osteopontin protein (score 0). Four of 6 squamous-cell carcinomas were immunopositive for osteopontin protein. These tumors also had the highest over-expression of osteopontin RNA (range 2.8 \times –4.5 \times). Immunopositivity was localized to squamous tumor cells throughout (scores 2–4), with only occasional or focal macrophage staining (score 1+, 4/6 tumors; 2+, 2/6 tumors). Immunopositivity in tumor cells was localized to the peri-nuclear region of the cell, whereas staining within macrophages was localized to the cytoplasm.

Patterns of osteopontin protein immunoreactivity varied considerably for esophageal adenocarcinomas. In the majority of tumor sections (74%, 14/19), immunopositivity was localized to macrophages and ranged from focal (1+) to strong (4+) macrophage staining. By contrast, tumor cell staining (scores 2–4) was seen in only 26% (5/19) of adenocarcinomas, and immunoreactivity gener-

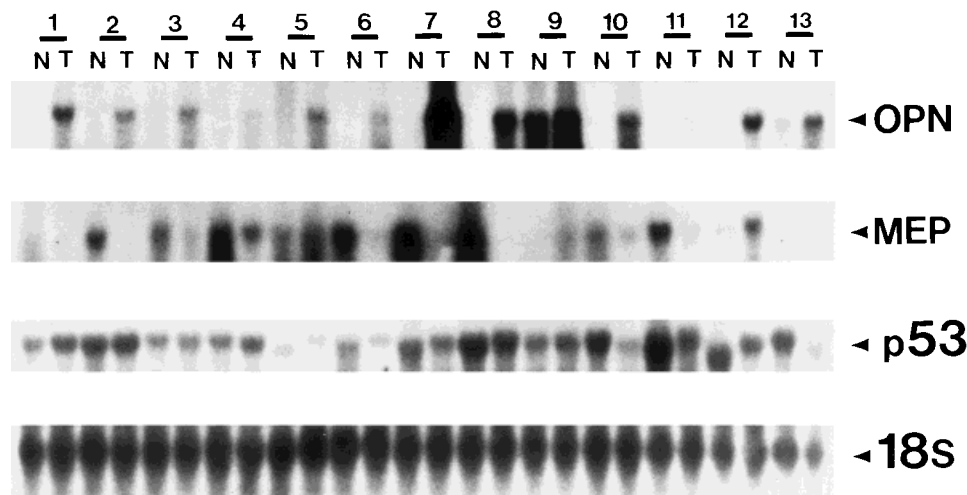


FIGURE 1 – Representative Northern blot analysis of 13 human esophageal tumors (T) and matched histologically normal esophageal mucosa (N) probed for expression of osteopontin (OPN), cathepsin L (MEP), p53 and an 18S rRNA control (on this blot, numbers 2, 5, 7 and 10 are esophageal squamous-cell carcinomas and numbers 1, 3, 4, 6, 8, 9, 11, 12, and 13 are primary esophageal adenocarcinomas).

TABLE I – EXPRESSION OF OSTEOPONTIN AND CATHEPSIN L, *ras* AND *p53* MUTATIONS IN HUMAN ESOPHAGEAL SQUAMOUS-CELL CARCINOMAS AND ADENOCARCINOMAS¹

Tumor		OPN RNA	OPN IHC		Cathepsin L RNA	<i>ras</i> mutation	<i>p53</i> mutation	p53 RNA	p53 protein
Stage	Diff		T	M					
Adenocarcinomas									
III	mod	8.7	2	3+	1.5	—	—	0.8	0
IV	poor	7.0	0	1+	1.1	—	5/158 CGC → CAC	0.5	0
III	poor	5.6	0	4+	4.5	K12 GTT	—	0.8	8
III	poor	5.1	2	1+	0.7		—	6/196 CGA → TGA	0.7
III	poor	5.0	0	2+	1.0	—	—	0.7	0
III	poor	4.5	0	1+	0.1	—	—	0.7	4
III	poor	3.6	0	3+	7.5	—	8/271 GAG → TAG	2.0	0
III	mod	3.3	0	2+	2.3	—	—	1.0	0
IIA	poor	3.2	0	0	3.9	—	—	1.5	0
III	well	2.6	0	3+	6.3	—	9/316 +C	1.0	0
IIA	well	1.9	0	0	3.0	—	—	2.5	0
III	poor	1.5	3	0	0.9	—	7/248 CGG → TGG	0.6	8
IIA	mod	1.2	3	4+	2.5	—	—	0.5	0
IIA	poor	1.1	0	0	1.7	—	7/234 TAG → AAC	0.6	6
III	poor	1.1	0	3+	1.6	—	5/158 CGC → CAC	0.1	8
IIA	poor	1.0	4	3+	0.7	—	5/175 CGC → CAC	1.2	8
III	poor	1.0	0	1+	1.7	—	5/175 CGC → CAC	1.6	8
III	poor	0.8	0	2+	4.3	—	—	0.5	0
III	poor	0.7	0	0	0.4	—	8/273 CGT → CAT	2.0	8
Squamous-cell carcinomas									
IIA	well	4.5	4	1+	1.0	—	5/173 GTG → TTG	0.8	7
IIA	mod	4.1	2	2+	1.4	—	8/293 +A	1.7	0
IIB	poor	3.9	4	2+	2.3	—	6/224 GAG → GTG	0.5	0
IIA	mod	2.8	3	1+	0.3	—	—	0.9	0
III	poor	2.2	0	1+	4.6	—	5/158-160 -CGCGCCA	0.7	4
IV	mod	1.7	0	1+	0.2	—	4/106 -CTA	0.7	7

¹Tumor stage according to UICC TNM staging system (1987); Diff, tumor differentiation, poor, moderate (mod), or well-differentiated; OPN, osteopontin; RNA expression, fold increase for tumor relative to matched normal tissues (and to 18S rRNA level to control for RNA loading); OPN IHC, osteopontin immunohistochemistry scores for tumor cell (T) staining expressed as a composite score of percentage (0, none; 1, <1%; 2, 1–10%; 3, 11–33%; 4, 34–66%; 5, >67%) and intensity (0, none; 1, weak; 2, moderate; 3, strong) of staining (range: 0 [negative], 2–8 [positive]), and staining of macrophages (M) was scored subjectively (0, none; 1+, focal staining; 2+, 3+, 4+, subjective range of positive staining); *ras* mutation, K-*ras*/codon/mutation; *p53* mutation, exon/codon(s)/base change; p53, composite immunohistochemistry score for p53 protein accumulation (0, none; 2–8, range of cell nuclear staining) (Allred *et al.*, 1993).

ally did not correlate with osteopontin RNA expression by Northern analysis.

Cathepsin L RNA levels in esophageal tumors

Cathepsin L RNA levels in the same esophageal tumor and matched normal tissues also were assessed by Northern analysis and quantitative densitometry (Fig. 1). Expression of cathepsin L RNA in esophageal tumors, relative to matched normals, varied widely (range 0.1×–7.5×), with over-expression (greater than 1.5× in 33% (2/6, range 2.3×–4.6×) of squamous-cell carcinomas and in 58% (11/19, range 1.6×–7.5×) of adenocarcinomas. No association between expression of cathepsin L RNA and osteopontin RNA was noted.

ras mutations

PCR allele-specific oligonucleotide hybridization was used to evaluate all possible mutations in 3 codons of all 3 *ras* genes. One

esophageal adenocarcinoma was found to have a mutation (G-to-T transversion) at codon 12 of K-*ras*, confirmed by sequencing (Fig. 2). By Northern analysis, this poorly differentiated adenocarcinoma (UICC stage III) also over-expressed osteopontin (5.6×) and cathepsin L (4.5×) RNA relative to matched normal esophageal mucosa.

p53 mutations, RNA levels and protein accumulation

Alterations in the *p53* tumor-suppressor gene were studied in the same tissues. *p53* mutations, detected by PCR/SSCP analysis and confirmed by sequencing, were found in 83% (5/6) of squamous-cell carcinomas. These comprised 2 mis-sense mutations (single G-to-T base substitution, 1; in-frame deletion, 1) and 3 truncating mutations (base deletion, 1; base insertion, 1; splice site alteration, 1). *p53* mutations were found in 53% (10/19) of primary esophageal adenocarcinomas, comprising 7 mis-sense mutations and 3

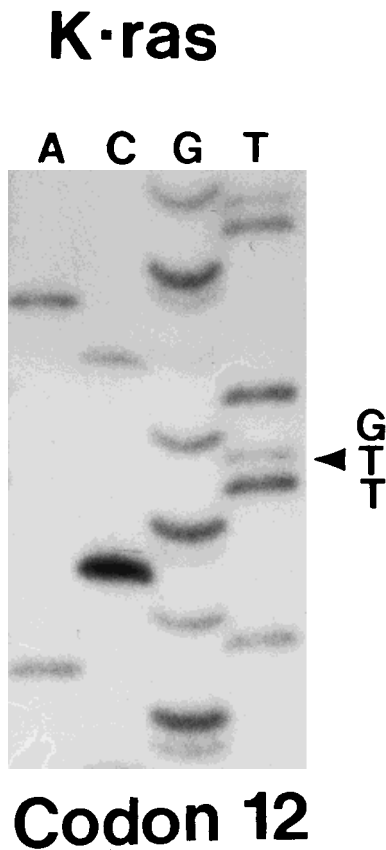


FIGURE 2 – Sequence analysis of a primary esophageal adenocarcinoma confirmed to have a mutation in codon 12 of the K-*ras* gene (GGT → GTT).

truncating mutations. Single-base substitutions were G:C-to-A:T ($n = 7$), G:C-to-T:A ($n = 1$) and A:T-to-T:A ($n = 1$), and 7 of these changes were at CpG dinucleotides. PCR/SSCP analysis of matched normal esophageal mucosae demonstrated no mutations (confirmed by sequencing random normals), indicating that *p53* mutations were of somatic origin.

Only 4 tumors (adenocarcinomas, 3; squamous-cell carcinoma, 1) over-expressed *p53* RNA relative to matched normal tissue (range $1.6\times$ – $2.5\times$). Three tumors had associated *p53* mutations (truncating, 2; mis-sense, 1), and the tumor with a mis-sense mutation was immunopositive. The adenocarcinoma with the highest over-expression of *p53* RNA ($2.5\times$) did not have a *p53* mutation and was immunonegative.

p53 protein accumulation was found in 50% (3/6) of squamous-cell carcinomas and 37% (7/19) of adenocarcinomas. Consistent immunoreactivity was seen using either the monoclonal (D07) or polyclonal (CM1) anti-*p53* antibody. Cell nuclear staining was characteristic, but staining intensity varied for individual cell nuclei. Although *p53* protein distribution was heterogenous throughout tissue sections, the composite score (evaluating both intensity and percentage immunoreactivity) was ≥ 5 for immunopositive tumors. No staining was seen in any negative control, matched normal esophageal mucosa or the cytoplasm of tumor cells. Concordance between independent investigators interpreting the slides was found in over 90% of cases. The association between immunoreactivity and *p53* mutations was as follows: 9 of the 10 immunopositive tumors had associated mis-sense mutations, 1 immunopositive tumor was found to have a truncating mutation (7-bp deletion, exon 5) and all other tumors ($n = 5$) with truncating *p53* mutations were immunonegative.

Associations between molecular alterations and clinico-pathologic findings

Molecular alterations (over-expression of osteopontin, cathepsin L or *p53* RNA; *ras* or *p53* mutations) were not associated with clinico-pathologic findings in this series.

DISCUSSION

In this series of 25 surgically resected esophageal carcinomas, using immunohistochemistry and Northern blotting of RNA, we have characterized the expression of 2 *ras*-regulated genes, whose products, osteopontin and cathepsin L, are associated with tumor invasion and metastasis. We demonstrated over-expression of both osteopontin and cathepsin L in squamous-cell carcinomas and adenocarcinomas of the esophagus. We then performed a comprehensive study of the same esophageal tissues for evidence of *ras* oncogene activation and found a single K-*ras* mutation in a poorly differentiated adenocarcinoma, which has not been reported so far in a human esophageal cancer. This novel finding was contrasted to the spectrum of *p53* gene alterations in the same series of tumors.

The role of the *ras* family of oncogenes in human esophageal tumorigenesis is unclear. Experimentally, carcinogen-induced rat esophageal tumors were found to have a high frequency of activating H-*ras* mutations (Wang *et al.*, 1990; Lozano *et al.*, 1994), which were predominantly G-to-A transitions, and the finding of *ras* mutations in associated esophageal papillomas suggested that activation of this gene occurs relatively early in the rat esophageal cancer model. Based on these experimental observations and reports of frequent *ras* mutations in human gastrointestinal and related upper aerodigestive tract (oropharynx and lung) malignancies, several groups have studied human esophageal carcinomas from different geographic regions but detected no *ras* mutations. Despite this surprising finding, other evidence has emerged to suggest a role for *ras* in human esophageal tumorigenesis. While one early study reported *ras* p21 protein, detected immunohistochemically, in esophageal squamous-cell carcinomas (Ruol *et al.*, 1990), other studies reported over-expression of H-*ras* RNA (Sorsdahl *et al.*, 1994) and K-*ras* amplification (Galiana *et al.*, 1995) in esophageal adenocarcinomas. Galiana *et al.* (1993) also reported *ras* mutations in 3 highly tumorigenic cell lines, derived from Japanese patients with squamous-cell carcinomas of the esophagus. Furthermore, transfection of a mutated H-*ras* gene into non-tumorigenic esophageal cell lines resulted in tumorigenesis. We report a *ras* mutation (K12, GGT-to-GTT) in a human esophageal cancer. This tumor was a poorly differentiated adenocarcinoma of primary esophageal origin (using strict criteria; Casson *et al.*, 1991). Although the majority of studies to date have evaluated only esophageal squamous-cell carcinomas, the frequency of *ras* mutation (5%, 1/19) in esophageal adenocarcinomas also appears to be very low.

This study also evaluates the expression of 2 *ras*-regulated genes in human esophageal cancer, whose products (osteopontin and cathepsin L) have previously been shown to be associated with tumor invasion and metastasis. Using Northern blotting of RNA extracted from fresh esophageal tissues, over-expression ($>1.5\times$) of osteopontin was demonstrated in 58% (11/19) of esophageal adenocarcinomas and 100% of squamous-cell carcinomas, relative to matched histologically normal esophageal mucosa. Immunohistochemistry was used to assess tissue distribution of osteopontin. Although this series evaluated only a limited number of squamous-cell carcinomas, osteopontin expression appeared to be localized to the tumor cells (rather than tumor-associated macrophages) and intensity of staining generally correlated with RNA expression. Patterns of osteopontin immunoreactivity varied considerably for esophageal adenocarcinomas, with greater localization of osteopontin to the infiltrating macrophages than to individual tumor cells. Little correlation was seen between levels of osteopontin RNA expression detected immunohistochemically or by Northern analysis in adenocarcinomas. We have reported a similar distribution of

osteopontin protein (*i.e.*, predominant localization in tumor-infiltrating macrophages rather than tumor cells) in non-small-cell lung cancers (Chambers *et al.*, 1996).

We also used Northern blot analysis to study expression of the MEP gene product in the same tumors. Over-expression of cathepsin L was found in 58% (11/19) of esophageal adenocarcinomas and 33% (2/6) of squamous-cell carcinomas, with wide variations in expression in both histologic sub-types. Interestingly, the adenocarcinoma with the *K-ras* mutation over-expressed both osteopontin (5.6 \times) and cathepsin L (4.5 \times) RNA. Our findings suggest that elevated levels of osteopontin and cathepsin L in human esophageal cancers contribute to the biology of this disease and that expression of these genes is mediated by *ras*-independent mechanisms in the majority of esophageal carcinomas.

The infrequency of *ras* oncogene mutations contrasts with the higher frequency of mutations of the *p53* tumor-suppressor gene in the same series of esophageal tumors. *p53* mutations were found throughout exons 4–10 studied in 53% (10/19) of adenocarcinomas and 83% (5/6) of squamous-cell carcinomas. As reported by previous studies (Montesano *et al.*, 1996), we found a high frequency of G:C-to-A:T transitions in esophageal adenocarcinomas. Such mutations, occurring at CpG dinucleotides, suggest that *p53* mutations occur spontaneously by deamination or mis-match repair in esophageal adenocarcinomas, in contrast to the spectrum of non-CpG mutations seen in esophageal squamous-cell carcinomas. We also used Northern blot analysis to evaluate expression of *p53* RNA and immunohistochemistry to study *p53* protein accumulation. To overcome the potential limitations of immunohistochemistry, we evaluated multiple tissue sections, used 2 anti-*p53* antibodies in independent assays and employed a scoring system to account for tissue heterogeneity, based on intensity of cell nuclear immunoreactivity and percentage staining. Furthermore, each immunohistochemical assay was repeated by the same laboratory technician at least twice and by a different technician at another research center. Consistent results were obtained by investigators evaluating the slides, who were also blinded to the results of the molecular analysis. As expected, *p53* protein was not detected immunohistochemically in all tumors with *p53* mutations, though good agreement was seen with mis-sense mutations.

Invasive carcinoma of the esophagus is associated with a dismal prognosis. Despite advances in multi-modality therapy, 5-year survival rates remain generally below 10%. While improvements in survival may be anticipated with early detection and rational use of adjuvant therapy, significant progress with this disease will likely only occur with an improved understanding of its tumor biology. To date, investigators have generally focused on the molecular genetic alterations associated with the early stages of esophageal tumor development and progression, including mutations of the *p53* tumor-suppressor gene, in pre-malignant (Barrett's) tissues (Cas-

son *et al.*, 1991). Although most patients with esophageal cancer present clinically with advanced disease, few studies have evaluated the molecular genetic events associated with the later stages of esophageal tumorigenesis or metastasis. As the *ras*-regulated genes osteopontin and cathepsin L have been implicated in tumor invasion and metastasis, we conducted this exploratory study utilizing fresh esophageal tissues obtained at surgery.

To avoid degradation of RNA, all esophageal tissues were processed immediately following esophagectomy by the same surgeon/investigator, and consequently, all Northern blots were of consistently good quality. Furthermore, no patient in this series had pre-operative chemotherapy or radiotherapy, which reflects the pattern of practice in Canada at the time of tumor banking. We also used strict clinico-pathologic criteria to define adenocarcinomas of primary esophageal origin, thereby excluding proximal gastric "cardia" cancers, and each tumor was staged (UICC, TNM) carefully at the time of resection in collaboration with a consultant pathologist. The prevalent histologic sub-type in this study was adenocarcinoma. Although this may reflect the increasing incidence of esophageal adenocarcinomas reported in North America, it also may represent patterns of referral to a tertiary or university thoracic surgical service. As patients in this study originated from a low-incidence geographic region, the relatively limited sample size precluded further statistical analysis of these data. Further studies, therefore, are warranted to confirm our observations in a larger series of esophageal tumors from other centers and to determine the roles of osteopontin, cathepsin L and *ras* in the development and progression of human esophageal cancer.

In summary, we report over-expression of osteopontin and cathepsin L in human esophageal cancer, implicating these gene products in esophageal tumorigenesis. We also report a *ras* mutation in a primary esophageal adenocarcinoma and contrast the frequency of these molecular changes with the spectrum of *p53* alterations in the same series of well-characterized esophageal tumors.

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