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Expression of Jun family members in human colorectal adenocarcinoma

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The products of the Jun family genes, c-Jun, JunB and JunD, are essential components of the activating protein-1 transcription factor complexes that are critically important in the control of cell growth, differentiation and neoplastic transformation. Although increased c-Jun expression has been reported in human colorectal tumors, expression of JunB and JunD in these tumors has not previously been characterized. In the current study, we examined 24 cases of human colorectal adenocarcinoma by western immunoblotting analysis and immunohistochemical staining for the expression of c-Jun, JunB and JunD proteins. Normal-appearing colonic mucosa distant from the tumors in the same colectomy specimens were used as a reference for comparison. The results showed that both c-Jun and JunB proteins were undetectable or barely detectable in normal mucosa but their expression levels were significantly increased in human colorectal adenocarcinomas. In contrast, JunD protein was present at high levels in normal mucosa and only showed a minimal increase in adenocarcinomas. These observations suggest that different Jun proteins may serve different roles in regulating colonic epithelial cell growth and in colorectal tumorigenesis.

Introduction

Colorectal tumorigenesis is a multistep process involving genetic alterations in both oncogenes and tumor suppressor genes (1–3). These include activating mutations in *ras* oncogenes seen in nearly 50% of colorectal adenocarcinomas (1,2) and loss-of-function mutations in the adenomatous polyposis coli (APC) tumor suppressor detected in >80% of human sporadic colorectal neoplastic epithelial lesions (4). Many studies have demonstrated that activated *ras*-induced neoplastic transformation is mediated through activation of a group of nuclear regulatory proteins, the activating protein-1 (AP-1) transcription factors, which are primarily composed of two protooncogene families, Jun and Fos (5,6). The members of the Jun family, c-Jun, JunB and JunD, function by forming either homodimers or heterodimers among themselves or with Fos proteins to regulate the transcriptional activity of AP-1-dependent genes (5–7). Although Jun–Fos heterodimers bind DNA at the AP-1 site more tightly and are more potent regulators of transcription than Jun–Jun homodimers, Fos proteins cannot form homodimers and thus cannot independently bind DNA (8). As a consequence, transcription of AP-1-dependent genes is primarily regulated by Jun protein expression levels since Jun proteins are absolutely required for AP-1 functioning (9).

Although c-Jun, JunB and JunD are closely related structurally and share many biochemical properties (5–7), they differ significantly in their biological functions. In many cell types, *c-jun* and *junB* are generally found to function as proliferation-promoting genes and their activation (especially *c-jun*) is required for cell cycle progression and neoplastic transformation (9–13). In contrast, the function of JunD is much less clear and several studies have suggested that it may actually function to inhibit cell proliferation (14–16). In this regard, it is significant that *c-jun*, but not *junB* or *junD*, has recently been shown to be a target gene of the β-catenin/T cell factor (Tcf) complex whose activity is negatively regulated by the APC tumor suppressor (17).

Only a few studies have examined c-Jun expression in human colorectal tumors (18,19) and no study has investigated the expression of JunB and JunD in these tumors. In the experiments described in this report, the expression levels of all three Jun proteins were studied in human colorectal adenocarcinomas by western immunoblotting analysis and immunohistochemical staining. The results show that both c-Jun and JunB are upregulated in colorectal carcinomas compared with normal colonic mucosa, whereas JunD expression levels are relatively unaffected by the process of colorectal tumorigenesis.

Abbreviations: AP-1, activating protein-1; APC, adenomatous polyposis coli; Tcf, T cell factor.

Materials and methods

Patient samples

Fresh tissue samples from 24 primary sporadic human colorectal adenocarcinomas were collected. Histologic examination showed five tumors to be well differentiated, 15 moderately differentiated and four poorly differentiated. Tumor size ranged from 2.5 to 11 cm (mean size 4.9 cm). Thirteen patients had regional lymph node metastases and seven had distant metastases mostly to the liver. Three of the tumors exhibited mucinous differentiation. Normal-appearing mucosa distant from the tumor (at least 5 cm from the edge of the tumor) in the same colectomy specimens were collected as controls. The fresh tissue samples removed from the specimens were immediately snap-frozen in liquid nitrogen and stored at –80°C until analysis. Precautions were taken during sampling to avoid necrotic or hemorrhagic areas and to minimize stromal contamination.

Preparation of soluble cell lysates

Frozen tissues were washed twice in 4°C phosphate-buffered saline (pH 7.4) and homogenized in 0.3–0.7 ml of 4°C lysis buffer that contains 50 mM Tris–HCl (pH 7.4), 120 mM NaCl, 0.5% Triton X-100 and 10 μg/ml phenylmethylsulfonyl fluoride. After sonication for 20 s, the tissue samples were centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was collected, aliquoted and frozen at –80°C before use. Protein concentrations were determined by using a protein assay kit obtained from Bio-Rad Laboratories (Hercules, CA) according to the supplier's instructions.

Western immunoblotting analysis

Fifty micrograms of cellular protein from human tissues were mixed with SDS sample buffer, boiled for 10 min and then subjected to electrophoresis on 10% SDS–polyacrylamide gels. Western immunoblotting procedures were performed as described previously (16). After the transfer of the proteins onto nitrocellulose filters, immunological evaluation was performed using an ECL western blotting analysis kit (Amersham Corp., Arlington Heights, IL) with

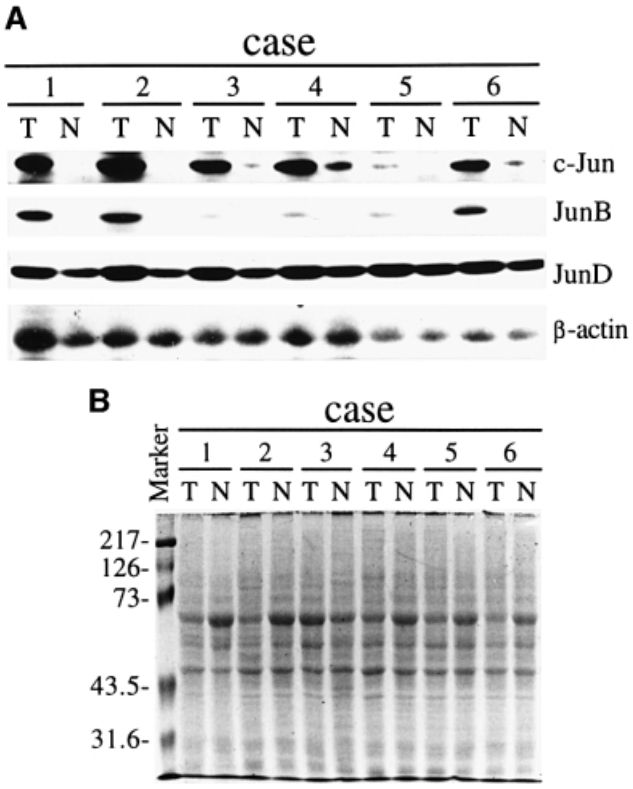

Fig. 1. Expression of c-Jun, JunB and JunD in human colorectal adenocarcinomas (T) and paired normal control mucosa (N) from six representative cases determined by western immunoblotting analysis (**A**). Fifty micrograms of soluble protein from each tissue sample were used. The expression levels of β-actin were used as loading controls. Equal protein loading was also monitored by staining the protein gels with Coomassie blue as exemplified in (**B**). Positions of molecular mass markers (in kDa) are shown to the left.

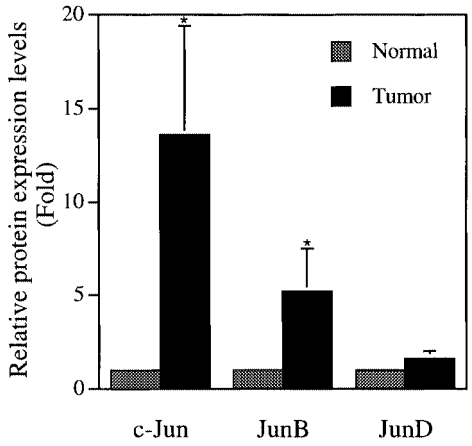

Fig. 2. Comparison of c-Jun, JunB and JunD expression in adenocarcinomas and normal control mucosa from 24 cases. The protein expression levels determined by western immunoblotting analysis were densitometrically quantitated and normalized with the expression levels of β-actin. The data are presented as means ± SD of relative fold expression where the expression levels of c-Jun, JunB and JunD in normal control mucosa are normalized to 1. *$P < 0.05$ by paired *t*-test.

rabbit polyclonal antibodies against c-Jun (N), JunB (N-17) or JunD (329) obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), or a mouse monoclonal antibody against β-actin (AC-15) obtained from Sigma Chemical Co. (St Louis, MO). The signals generated by chemiluminescence on the X-ray films with linear exposure were quantitated by densitometric analysis.

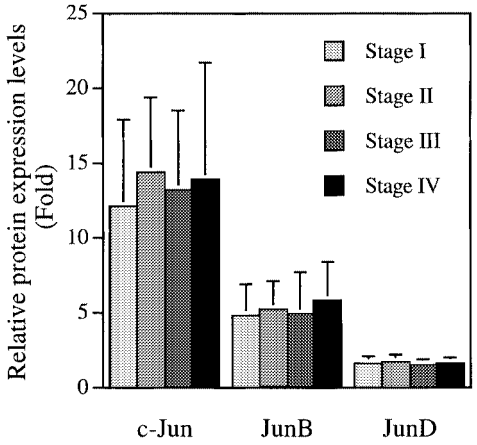

Fig. 3. Lack of correlation of c-Jun, JunB and JunD expression levels with clinical stages. The protein expression levels determined by western immunoblotting analysis were densitometrically quantitated and normalized with the expression levels of β-actin. The cases were divided into four groups according to the TNM staging system: stage I ($n = 4$), stage II ($n = 7$), stage III ($n = 6$) and stage IV ($n = 7$). The data are presented as means ± SD of relative fold expression using the expression levels in normal colonic mucosa as a reference (designated as 1).

Immunohistochemistry

Immunohistochemical staining was performed using the DAKO LSAB2 horseradish peroxidase system and DAKO AEC substrate system (DAKO Corp., Carpinteria, CA) according to the manufacturer's instructions with slight modifications. Briefly, deparaffinized tissue sections were first treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase followed by incubation with blocking serum for 20 min. The sections were then incubated with primary rabbit polyclonal antibodies against c-Jun, JunB or JunD at room temperature for 1 h at final antibody concentrations of 2 μg/ml diluted in blocking serum solution. After further incubation with biotinylated link antibody and peroxidase-labelled streptavidin, the staining was developed by reaction with AEC substrate–chromogen solution followed by counterstaining with hematoxylin. In each experiment, a negative control was included where the primary antibodies for c-Jun, JunB or JunD were replaced by a non-human reactive rabbit IgG (Santa Cruz Biotechnology, Inc.).

Results

Twenty-four primary sporadic human colorectal adenocarcinomas were examined for the expression of c-Jun, JunB and JunD proteins by western immunoblotting analysis in comparison with paired normal colonic mucosa using soluble cell lysates prepared from surgical colectomy specimens. Shown in Figure 1A are six representative cases, which demonstrated that in normal control mucosa the expression levels of c-Jun and JunB were essentially undetectable or barely detectable, but their levels were significantly increased in colorectal carcinomas. In contrast, JunD protein was present at relatively high levels in normal mucosa and was only modestly elevated in carcinomas. Similar results were also observed with lysates prepared from 18 other cases, with all tumors exhibiting elevated c-Jun and JunB protein levels relative to control normal mucosa. In these experiments, the protein levels of β-actin were also examined as loading controls. However, since the β-actin levels varied somewhat among different cases (Figure 1A), protein loading was also monitored by staining the protein gels with Coomassie blue as shown in Figure 1B.

Figure 2 summarizes the quantitative data from 24 cases studied by western immunoblotting analysis and shows that, compared with normal colonic mucosa, adenocarcinomas showed ~13- and ~5-fold increases in the expression levels of c-Jun and JunB, respectively ($P < 0.05$ by paired *t*-test). The

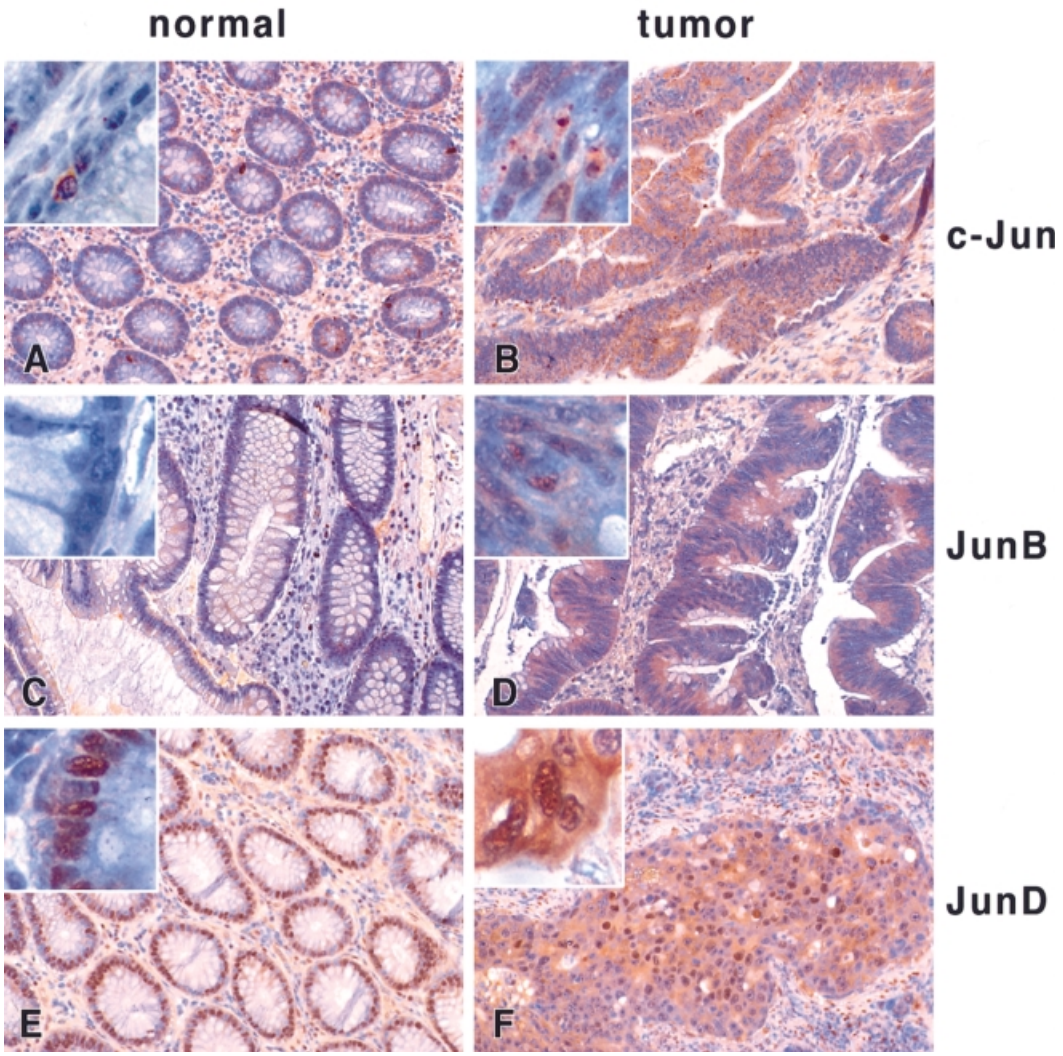

Fig. 4. Comparison of c-Jun, JunB and JunD expression in human colorectal adenocarcinoma and normal control mucosa by immunohistochemistry (original magnification ×85). Higher power views are highlighted in inserts (original magnification ×470). Six cases were studied with similar results.

expression levels of JunD in adenocarcinomas only showed ~1.5-fold increase, which was statistically insignificant ($P > 0.05$). Interestingly, the abundance of c-Jun and JunB proteins varied significantly among different tumors, but no consistent correlation was observed between these two proteins. In more than one-third of the tumors examined, the increase in JunB expression levels was not proportionate to that of c-Jun (see Figure 1A for examples).

There was no correlation between clinical TNM stages of the tumors and the expression levels of any of the three Jun proteins (Figure 3). Other pathologic features, such as tumor size, the degree of histologic differentiation or mucin production, also did not correlate with the expression levels of c-Jun, JunB or JunD. The TNM staging system is defined as follows: T, the extent of the primary tumor; N, the absence or presence of regional lymph node metastasis; M, the absence or presence of distant metastasis.

Immunohistochemical staining confirmed immunoblotting results and demonstrated that Jun protein expression is largely restricted to the neoplastic epithelial components of the tumors (Figure 4). Normal mucosa showed comparatively weaker staining than the tumor cells. More specifically, immunostaining for c-Jun revealed only scattered epithelial cells in the normal mucosa to be weakly positive in the nucleus and immunostaining for JunB was essentially negative, in contrast to a more uniform and stronger nuclear staining seen in the neoplastic cells (Figure 4, see inserts for higher magnification). In addition, an altered subcellular distribution of JunD protein was noted in tumor cells, in that both nucleus and cytoplasm were diffusely stained (Figure 4F), whereas in normal mucosa JunD was primarily localized to the nucleus (Figure 4E).

Discussion

Regulation of the Jun/AP-1 family genes has been proposed to be critically important in the control of cell growth and neoplastic transformation (5,6,15). For example, cells overexpressing *c-jun* were found to grow in low-serum-containing medium and to develop colonies in agar, whereas cells overexpressing *junB* grew in agar with a reduced efficiency but did not grow in low-serum-containing medium (10). Overexpression of *junD*, on the other hand, either had no

effect on cell growth (10) or had resulted in slower cell growth and an increase in the percentage of cells in G_0/G_1 phase of the cell cycle (14). JunD was also found to partially suppress neoplastic transformation induced by an activated *ras* oncogene in murine fibroblasts (14). In human ovarian tumors derived from surface epithelium, the expression levels of *junD* were found to be downregulated when compared with normal ovarian surface epithelial cells. In contrast, the expression levels of *c-jun* and *junB* were comparable with the normal cells although high *junB* levels in tumors seemed to correlate with a more malignant phenotype (20).

Our results presented in this report are in accord with the few published studies that have examined c-Jun expression in human colorectal tumors. Using immunohistochemistry, Magrisso *et al.* (18) found that the expression of c-Jun was infrequent in normal colonic mucosa but common in colorectal adenocarcinomas. Using western immunoblotting analysis, Pandey *et al.* (19) demonstrated elevated levels of c-Jun in colorectal adenocarcinomas and in adjacent histologically normal-appearing mucosa up to 4 cm from tumor margins. More importantly, our data provide further evidence to support the recent report (17) showing that *c-jun* is a downstream target gene controlled by the β-catenin/Tcf pathway as demonstrated by transfection assays using colorectal cell lines. This is so because in the same 24 tumors reported here (where c-Jun was found to be upregulated), we have also documented increased expression of β-catenin (compared with normal control mucosa), presumably as a result of APC and/or β-catenin mutations (21). On the other hand, our results suggest that upregulation of c-Jun expression in human colorectal carcinomas may not be entirely *ras*-dependent because the reported rate of *ras* mutations in colorectal tumors is $<50\%$ (1,2). In this regard, it has been shown recently that in rat intestinal tumors induced by 1,2-dimethylhydrazine, the presence of K-*ras* mutations did not correlate with the kinase activity of either c-Jun N-terminal kinase (JNK) or extracellular signal regulated kinase (ERK), two important positive regulators of the AP-1 activity (22). In that study, all the adenomas and carcinomas examined displayed elevated JNK and ERK activity but only 56% of the tumors contained a mutation in K-*ras*.

The expression of JunB in human colorectal adenocarcinomas has not previously been examined. In this report, we demonstrated for the first time that in addition to c-Jun, JunB is also upregulated in human colorectal carcinomas. This is not unexpected, however, since *junB*, like *c-jun,* is generally considered as a proliferation-promoting gene (10,12,15), although it may be less potent than *c-jun* in transforming cells (10). Whether *junB* is also regulated in a manner similar to *c-jun* in human colorectal neoplasms (i.e. controlled by the β-catenin/Tcf pathway and/or mutated *ras* gene) is currently unknown. However, the fact that the expression levels of JunB were not closely correlated with c-Jun levels suggests that while JunB induction is also characteristic of neoplastic colonic epithelium, its expression may be regulated independently of c-Jun in these cells.

In contrast to c-Jun and JunB, JunD expression appears to be regulated differently in human colorectal epithelium. Our results demonstrate that normal colonic epithelial cells express JunD at much higher level than c-Jun and JunB. Moreover, carcinoma cells show only a slight increase in JunD protein levels, in contrast to the marked increases seen in c-Jun and JunB. These findings are consistent with the current hypothesis that JunD may function to suppress cell growth (14).

In summary, we demonstrate that in addition to c-Jun, JunB is also markedly upregulated in human colorectal carcinomas. Both c-Jun and JunB proteins are essential components of the AP-1 transcription factor complexes that may serve important roles in mediating the transforming effects of β-catenin and/or mutated *ras*. JunD is present at relatively high levels in normal colonic epithelium and neoplastic transformation does not seem to alter its expression level drastically, suggesting that it may serve a role different from c-Jun and JunB in regulating colonic epithelial cell growth and colorectal tumorigenesis.

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