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Up-regulation of transcriptional factor E2F1 in papillary and anaplastic thyroid cancers

Received: 26 December 2003 / Accepted: 1 March 2004 / Published online: 29 April 2004
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Abstract Expression of genes in the Rb-E2F signaling pathway is controlled by E2F transcriptional factors originally defined as molecules that bind to the promoter of E2 adenovirus. The E2F gene family consists of six members and is designated E2F1–6. The Rb-E2F signaling pathway is among the main regulators of the cell cycle, hence its importance in differentiation and oncogenesis. We document here up-regulation of *E2F1*, but not other members of the E2F gene family, in 15 of 18 primary papillary thyroid cancers examined (83%) in comparison to corresponding noncancerous thyroid tissues and in all of 11 anaplastic thyroid cancer (ATC) cell lines (100%). The *E2F4* gene, however, was down-regulated in 12 of the papillary thyroid cancers (67%). Immunohistochemical analysis with antibody to *E2F1* revealed prominent intracellular *E2F1* protein in most of the primary papillary cancers (16 of 18; 89%) but was not detectable in normal thyroid tissues. These data indicated that increased expression of the *E2F1* gene might play a significant role in human thyroid carcinogenesis through derangement of the Rb-E2F signaling pathway.

Keywords E2F gene family · Papillary thyroid cancer · Anaplastic thyroid cancer · mRNA expression · Immunohistochemistry

Introduction

The Rb-E2F signaling pathway is an important regulator of cell cycle and differentiation, and its dysfunction can lead to oncogenesis. E2Fs are the main cellular targets of Rb protein (Chellappan et al. 1991), but Rb and E2F play opposite roles in the cell cycle: Rb-E2F complexes maintain cells in the G1 phase until Rb is phosphorylated after a growth signal induces activation of D Cyclins/cdk4. Phosphorylated Rb releases E2Fs from the complex, whereupon accumulated E2Fs activate downstream genes such as DNA polymerase alpha, Thymidine kinase 1, 2, Dhfr, and cdc6 that induce cells to S phase (see review, Nevins 2001). Disruption of the Rb/E2Fs signaling pathway, for example when the Rb gene is mutated, appears to play a critical role in cancer development and progression.

The E2F gene family was originally defined as encoding transcriptional factors that bind to the promoter sequence of the E2 adenovirus (Kovesdi 1987). E2Fs have since been found to exert multiple functions in regulation of the cell cycle. Some of them activate transcription in some circumstances but act as transcriptional regulators or repressors in others. The E2F transcriptional family consists of six members (*E2F1*, *E2F2*, *E2F3*, *E2F4*, *E2F5*, and *E2F6*). The promoter regions of genes in the Rb-E2F cascade contain an E2F-binding *cis* sequence, allowing E2Fs to control expression of downstream genes.

In the work reported here, we studied mRNA expression and carried out immunohistochemical analysis of all six members of the E2F gene family in human primary papillary thyroid cancers (PTC) and in cell lines derived from anaplastic thyroid cancers (ATCs). Our

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data implicated up-regulation of the *E2F1* gene as a specific event in thyroid tumorigenesis.

Materials and methods

Patient profile

Primary PTCs and adjacent normal thyroid tissues from 18 thyroid cancer patients who underwent surgery at Ito Hospital, Tokyo, from 1996 to 2000 were excised, frozen immediately, and stored at -80°C . All patients gave informed consent according to guidelines approved by the Institutional Research Board. Mean patient age at the time of surgery was 43.2; 16 were females. Clinico-pathological parameters were classified according to the International Union Against Cancer (UICC) staging system. The relationship between gene expression status and these clinical parameters was examined by chi-square test with Statview version 5.0 (SAS Institute, Inc., Cary, NC, USA).

Anaplastic thyroid cancer cell lines (ACL)

The 11 cell lines available for this study were derived from ATCs 8305c, 8505c, ARO, FRO, TTA1, TTA2, TTA3, KTA1, KTA2, KTA3, and KTA4. The cells were maintained in Dulbecco's modified eagle medium (Invitrogen, Carlsbad, CA, USA) for 8305c and 8505c; Minimum essential medium for ARO and FRO; and RPMI 1640 for TTA1, TTA2, TTA3, KTA1, KTA2, KTA3, and KTA4. All the media contained 10% fetal bovine serum without antibiotics. The cells were incubated in 37°C incubator with 5% CO_2 atmosphere.

RNA extraction

Frozen tissues were homogenized in the presence of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and total RNAs were extracted according to the manufacturer's instructions. In the case of cell lines, RNAs were extracted directly in the culture flasks, also with TRIzol reagent. One-microgram aliquots of the extracted RNAs were electrophoresed on 3.0% formaldehyde-denaturing gels in the usual manner to eliminate degenerated RNA. RNeasy Kits (QIAGEN, Valencia, CA, USA) were used to eliminate contaminating DNA.

cDNA synthesis

Using 5 g of each RNA as a template, cDNAs were synthesized in the usual manner. Briefly, the template mixed with 1 l of oligo dT₁₂₋₁₈ (Invitrogen, Carlsbad, CA, USA) used for annealing primer and denatured at 70°C for 10 min before addition of 200 U of reverse transcriptase II (Wako pure chemical, Tokyo, Japan) reaction buffer, 40 U of RNase inhibitor (Wako-Junyaku), and 10 mol dNTPs. This mixture was incubated at 42°C for 60 min after which the product was treated with 2 U of RNase (Wako-Junyaku) at 37°C for 20 min.

Semiquantitative RT-PCR

We performed semiquantitative RT-PCR (SQ-PCR) analysis to evaluate expression of E2F genes in our samples. Each 30- μl reaction mixture consisted of 1 μl of cDNA [concentration adjusted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ono et al. 2000)], 1 U of ExTaq (Takara, Ohtsu, Japan), 10 nM dNTPs, 1 $\times$ reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2), and 10 pmol each forward and reverse primers. For this procedure, sequence information for each gene was obtained from the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>), and primers (Table 1) were designed with the Primer 3 program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Conditions for the PCR experiments in the Gene Amp 9600 (Applied Biosystems, Foster City, CA, USA) were 94°C for 2 min for denaturing then 94°C for 30 s, $55-66^{\circ}\text{C}$ for 30 s, and 72°C for 30 s, repeated for 23–34 cycles. The products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The intensities of targeted bands were captured in 16-bit images and evaluated by the AlphaImager 3300 system (AlphaInnotech, San Leandro, CA, USA). To access the precise levels of gene expression, i.e., to evaluate band intensities at the logarithmic phase of amplification, bands that appeared to have reached a plateau were checked with AlphaImager software. If a saturated band appeared, the SQ-PCR was done again with reduced cycles. All SQ-PCR experiments were duplicated. In the case of cell lines, the controls for the SQ-PCR procedure consisted of normal cDNAs from five PTC patients.

Table 1 Primers used in this study

Gene	Accession no.	Forward primer ^a	Reverse primer ^a
<i>E2F1</i>	AF516106	ggggagaagtcacgctatga	ctcagggcacaggaaaacat
<i>E2F2</i>	NM_004091	ggccaagaacaacatccagt	ggctgtcagtagcctccaag
<i>E2F3</i>	NM_001949	gccctccagaacaagact	gggagcttttccaaatcgta
<i>E2F4</i>	AF527540	gtgccaccacctgaagattt	ggtggagaagacgaagcag
<i>E2F5</i>	Z78409	aaggtgtaggtgctggctgt	cagagcctggcttcttcag
<i>E2F6</i>	NM_001952	gttgcaacgaactgggagt	cttcttctcaggcgcttct

^aPrimer sequence was described 5' to 3' side (left to right).

Quantitative RT-PCR

In order to reconfirm the up-regulation of *E2F1* and down-regulation of *E2F4* in PTC, real-time quantitative RT-PCR (Q-PCR) was performed. Q-PCR was carried out with qPCR Mastermix for Syber Green I (Eurogentec, Seraing, Belgium) and ABI 7700 (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions with some modifications. Primers and PCR conditions for Q-PCR of *E2F1* and *E2F4* were the same as those used in the SQ-PCR study. The template of Q-PCR was the same as that used in SQ-PCR, which was adjusted for the amount of cDNA monitoring of GAPDH expression. Expression difference between normal thyroid tissue and PTC in case *X* was defined as follow: $\Delta C_{tX} = C_{t-PTCX} - C_{t-NormalX}$ (C_t , threshold cycles for target amplification), Expression difference (PTC/Normal) = $2^{(-\Delta C_t)}$.

Immunohistochemical analysis of *E2F1* expression

To evaluate the expression of *E2F1* in protein levels, we performed immunohistochemical analysis of primary tumor (PTC) samples using 4-μm thick sections of paraffin-embedded tissue. Monoclonal antibody for *E2F1* gene product (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) served as the primary antibody. Antigen retrieval in a microwave was employed prior to immunostaining with VECTASTAIN Elite ABC kits (Vector Laboratories, Inc., Burlingame, CA, USA) and Dako ENVISION kits (Dako Corporation, Carpinteria, CA, USA) following the manufacturers' instructions. After the sections were counterstained with hematoxylin, they were scanned at low power to identify areas that were evenly stained. Estimates of positive cells were scored as follows: 0%, negative; 1–10%, 1+; 11–25%, 2+; 26–50%, 3+; > 50%, 4+ (Saiz et al. 2002).

Results

Up-regulation of *E2F1* in PTCs

Using the semiquantitative RT-PCR (SQ-PCR) method, we examined paired normal and tumor DNAs from 18 patients with primary PTC for differences in expression of all six members of the *E2F* transcription-factor family (*E2F1*–6). The concentration of each RT product was equalized to that of GAPDH, a housekeeping gene chosen as a quantitative control. Representative SQ-PCR results for each *E2F* gene are presented in Fig. 1. Increased expression of *E2F1* is evident in tumors 6, 24, 25, 26, 27, 17, 14, and 15. Of the six genes, only the *E2F1* mRNA was up-regulated in a strong majority of the primary PTCs examined when tumor tissues were compared to their corresponding noncancerous tissues (15 of 18; 83%). Densitometric quantification showed that the

Fig. 1 Semiquantitative RT-PCR (SQ-PCR) images in representative primary papillary thyroid cancer (PTC) cases. *E2F1* showed prominent up-regulation in most primary tumors (T) compared with normal thyroid tissues (N). *E2F2* showed a tendency toward up-regulation but without statistical significance, while *E2F4* was down-regulated in all thyroid cancer tissues shown here (and in two thirds of the total examined). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for the RT-PCR experiments. *M* size marker

intensities of the *E2F1* bands in tumor samples were 2-to-10-fold greater than in bands representing normal thyroid tissues, but band intensities for the other five *E2F* genes were not significantly increased in tumors. However, the *E2F4* gene was down-regulated in two thirds of the primary tumors (12 of 18; 67%). There was no relationship between *E2Fs* gene expression status and clinicopathological parameters.

Quantitative RT-PCR for *E2F1* and *E2F4* in PTC

In order to confirm the up-regulation of *E2F1* and down-regulation of *E2F4* in PTC, real-time Q-PCR was performed. Expression of *E2F1* was remarkably increased 1.05-to-19.03-fold in PTC. On the contrary, *E2F4* was significantly decreased 0.03-to-0.68-fold in PTC compared to normal thyroid tissue in each tested cases. (Table 2) The result of SQ-PCR was confirmed by quantitative RT-PCR also.

Immunohistochemistry of *E2F1* protein in papillary thyroid cancers

Representative results of immunohistochemical staining of primary PTC specimens are shown in Fig. 2a,b; no *E2F1* expression was detectable in normal thyroid tissue (Fig. 2c). Of the 18 PTCs examined in the present study, the majority overexpressed *E2F1* protein (16 of 18; 89%) in accord with the up-regulation of *E2F1* mRNA in the same tumors.

Table 2 Results of quantitative RT-PCR of *E2F1* and *E2F4* in primary papillary thyroid cancer (PTC) cases. C_{t-PTC} , threshold cycles for PTC; $C_{t-Normal}$, threshold cycles for normal thyroid tissue; ΔC_t , $C_{t-PTC} - C_{t-Normal}$; ED, expression difference (PTC/Normal)

E2F1					E2F4				
Case	C_{t-PTC}	$C_{t-Normal}$	ΔC_t	ED	Case	C_{t-PTC}	$C_{t-Normal}$	ΔC_t	ED
24	31.53	35.78	-4.25	19.03	24	28.79	27.26	1.53	0.35
23	31.91	35.51	-3.6	12.13	23	29.46	26.85	2.61	0.16
7	33.41	34.68	-1.27	2.41	7	30.31	27.99	2.32	0.20
20	30.76	34.19	-3.43	10.78	20	29.14	25.62	3.52	0.09
22	32.96	36.12	-3.16	8.94	22	29.55	27.25	2.3	0.20
5	32.05	34.22	-2.17	4.50	5	29.98	26.97	3.01	0.12
6	30.2	34.15	-3.95	15.45	6	30.24	27.07	3.17	0.11
14	33.55	36.17	-2.62	6.15	14	31.81	28.57	3.24	0.11
2	33.07	36.05	-2.98	7.89	2	30.66	27.38	3.28	0.10
10	33.66	35.28	-1.62	3.07	10	31.06	28.41	2.65	0.16
25	33.11	35.88	-2.77	6.82	25	28.99	26.97	2.02	0.25
26	30.01	33.98	-3.97	15.67	26	27.05	24.31	2.74	0.15
27	32.98	34.63	-1.65	3.14	27	27.93	25.39	2.54	0.17
17	33.06	34.12	-1.06	2.08	17	31.26	28.11	3.15	0.11
15	32.86	34.42	-1.56	2.95	15	32.44	27.3	5.14	0.03
1	32.02	32.09	-0.07	1.05	1	29.57	29.01	0.56	0.68
2	34.21	36.88	-2.67	6.36	2	32.01	31.32	0.69	0.62
9	32.93	34.17	-1.24	2.36	9	32.55	31.38	1.17	0.44

Fig. 2a-c Immunohistochemical analysis of E2F1 expression in primary papillary thyroid cancer (PTC). **a** Low-power ($\times 40$) view of immunostained E2F1; E2F1 protein expression was confirmed in the cancer cells. **b** High-power ($\times 200$) view of the same sample. **c** Normal thyroid gland

Up-regulation of *E2F1* in ATCs

Since most patients affected with ATC are treated with nonsurgical methods, such as chemotherapy and/or radiation, fresh surgical specimens for that type of cancer are rarely available. Therefore, we examined all six E2F genes in 11 cell lines that had been established from ATCs. SQ-PCR experiments revealed increased expression of *E2F1* mRNA in all 11 lines (Fig. 3). No significant increase was observed for any of the other E2F genes. The reduction in expression of the *E2F4* gene we detected among papillary tumors was not observed in the ATC cell lines.

Discussion

Thyroid cancers are classified as either medullary, papillary, follicular, or anaplastic. Medullary cancer derives

Fig. 3 Representative images of SQ-PCR experiments using anaplastic thyroid cancer (ATC) cell lines. *N1-N5* cDNA from normal thyroid gland, *C1* 8305c, *C2* 8505c, *C3* ARO, *C4* FRO, *C5* TTA1, *C6* TTA2, *C7* TTA3, *C8* KTA1, *C9* KTA2, *C10* KTA3, *C11* KTA4, *M* size marker

from parafollicular C cells; papillary, follicular, and anaplastic cancer originate from follicular cells of the thyroid gland. Anaplastic cancers are thought to arise mainly from a background of differentiated (papillary or follicular) cancer on the basis of clinicopathological observations that they are often accompanied by differentiated cancer cells and because they often arise in patients who had previously been treated for differentiated cancer of the thyroid. PTC is the most common type, accounting for about 80–90% of all thyroid cancers in Japan. It has a fair prognosis among thyroid cancers. A characteristic chromosomal rearrangement that gives rise to several types of chimeric *RET* genes has been described in a minority of PTCs (Onda et al. 2001). Although abnormalities of *RET*, *PTEN*, *p53*, *Rb*, and microsatellite-instability genes have been described in PTCs (Chiappetta et al. 2002; Dahia et al. 1997; Holm and Nesland 1994; La Perle et al. 2000; Onda et al. 2001, 2002; Puglisi et al. 2000), the mechanism of carcinogenesis in this type of cancer is largely unknown.

The clinical behavior of ATC is markedly distinct from other types of thyroid carcinoma. Among other things, it is one of the most virulent of all human malignancies; mean survival time among patients is less than 1 year after diagnosis, regardless of treatment mode (Wiseman et al. 2003). Differences in biological characteristics among thyroid tumors might be explained by variations in the pattern of genetic alterations among genes that participate in mechanisms of growth and differentiation, but the molecular mechanism underlying ATC is poorly understood.

The retinoblastoma (Rb) tumor-suppressor pathway involves several downstream effectors including p107, p130, p21, and E2F transcriptional factors (Dyson 1998; Nevins 2001). Among them, the E2Fs are essential for growth regulation by Rb protein. The hypophosphorylated form of Rb binds to E2Fs during the G1 phase (Chellappan et al. 1991), and this relationship inhibits the transcriptional activity of E2Fs (Flemington et al. 1993). The Rb-E2F complex actively represses E2F target genes by recruiting histone-modifying enzymes to their promoters (Magnaghi-Jaulin et al. 1998). To signal cell growth, Rb is phosphorylated by the cyclin-dependent kinases (Buchkovich et al. 1989; DeCaprio et al. 1989), whereupon transcriptionally active E2Fs are released from the complex at the G1/S transition. In this way, E2Fs contribute to either activation or repression of their target genes (Cloud et al. 2002). Disruption of various parts of the pathway that controls accumulation of E2F molecules, such as mutation of *Rb*, lead to loss of growth control and development of various human cancers (Saavedra et al. 2002).

Each member of the E2F family has a unique role at the protein level. *E2F1* not only acts as a growth-promoting factor (Gorgoulis et al. 2002; Yamasaki et al. 1998; Zhang et al. 2000) but also promotes apoptosis depending on the type of cell and the status of the upstream *Rb* gene (DeGregori et al. 1997). *E2F2* is a positive regulator of transcription (Schlisio et al. 2002);

however, loss of *E2F2* in Rb (–/–) embryos partially reduces the unscheduled DNA replication without affecting apoptosis (Saavedra et al. 2002). *E2F3* also has a unique character in cell-cycle regulation: In mouse embryonic fibroblasts, it acts in a dose-dependent manner to regulate mitogen-induced transcriptional activation of known E2F target genes (Humbert et al. 2000). Like *E2F1*, *E2F3* contributes both to apoptosis and to the inappropriate cell proliferation resulting from loss of Rb (Ziebold et al. 2001). In addition, *E2F3*-deficient mice suffer premature death from congestive heart failure (Cloud et al. 2002). *E2F4* is the most abundant of the E2Fs, comprising the largest moiety of E2F protein in most cells (Ikeda et al. 1996; Moberg et al. 1996); its overexpression induces serum-starved rat embryo fibroblasts to enter S phase and fails to induce apoptosis in the affected cells (DeGregori et al. 1997; Lukas et al. 1996). *E2F5* binds to Rb proteins, and homozygous *E2F5* knockout mice develop hydrocephalus as a result of excessive cerebrospinal fluid (Lindeman et al. 1998). *E2F6*, however, lacks transcriptional activation and Rb-binding domains; this gene product apparently inhibits E2F-dependent transcription that is independent of the Rb-E2F complex (Morkel et al. 1997).

In the present study, we demonstrated that *E2F1* mRNA was up-regulated in nearly all papillary and ATC specimens we examined. Up-regulation of the *E2F1* gene in primary PTCs was further evidenced by increased amounts of E2F1 protein in the tumor cells, shown by immunohistochemical staining with anti-E2F1 antibody.

E2F1 works as a critical “cellular-decision fork” (Wyllie 2002). Cells transfected with *E2F1* and *DP-1* induced tumors in nude mice (Johnson et al. 1994), and increased E2F1 activity can induce skin tumors in p53-null mice (Pierce et al. 1998). Expression of *E2F1* mRNA is associated with keratinocyte proliferation (Jones et al. 1997). At the genomic level, E2F1 was amplified 8-to-12-fold in the HEL erythroleukemia cell line (Saito et al. 1995), suggesting that genomic amplification of *E2F1* confers a growth advantage on those tumor cells. On the other hand, the apoptosis-inducing capacity of *E2F1* was demonstrated in TgT₁₂₁ E2F1^{–/–} mice, where apoptosis was inhibited by 80% and tumor growth was not accelerated, although it was clear that E2F1 was required for efficient proliferation of the tumor cells (Pan et al. 1998). In mouse embryos mutant for both *Rb* and *E2F1*, apoptosis and S-phase entry were significantly suppressed (Tsai et al. 1998). Given these contradictory effects of *E2F1*, it appears that activation of *E2F1* stimulates signals for either proliferation or apoptosis, the specific cellular conditions determining which outcome will be favored. Our results with PTCs and ATC cell lines suggested that in thyroid tumors, *E2F1* acts in its capacity as a proliferative force. With respect to tumorigenesis of thyroid tissues, others have shown that loss of *E2F1* significantly reduces the frequency of hyperplastic lesions in the thyroid glands of

Rb+/- mice (Yamasaki et al. 1998). Our results, together with that report, suggest that *E2F1* makes a tissue-specific contribution in the thyroid gland.

In the present study, *E2F4* tended to be down-regulated in papillary thyroid cancers but not in anaplastic cancer cell lines. Others have shown that *E2F4* is necessary for pocket protein-mediated arrest of cycling cells in the G1 phase; it may have a "stop" function for the E2F family (Gaubatz et al. 2000). Given the down-regulation of *E2F4* and up-regulation of *E2F1* in our primary thyroid cancer samples, both genes might play important roles in thyroid carcinogenesis.

In conclusion, the results reported here suggest that up-regulation of *E2F1* is involved in thyroid carcinogenesis, supported by the evidence of overexpression of *E2F1* mRNA and the presence of E2F1 antigen in PTCs. This information might contribute to the development of a molecular-targeted therapy for thyroid cancers that show aberrant expression of the *E2F1* gene.

Acknowledgments The authors wish to thank Kyoko Shimizu, Junko Sato, Mayumi Tanaka, and Naoko Tsuruta for their secretarial assistance. This work was supported by special grants for Strategic Advanced Research on "Cancer" from the Ministry of Education, Science, Sports and Culture of Japan; by a Research Grant from the Ministry of Health and Welfare of Japan; and by a Research for the Future Program Grant of The Japan Society for the Promotion of Science.

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