EXPERIMENTAL STUDY

The highly malignant phenotype of anaplastic thyroid carcinoma cell lines is recessive

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Abstract

Objective: The aim of our studies was to determine whether the phenotype of the anaplastic thyroid carcinomas is dominant or recessive. In fact, it is hypothesized, on the basis of epidemiological and pathological data, that undifferentiated thyroid carcinomas are derived from differentiated tumours through a mechanism of tumour progression.

Design: Cell hybrids have been generated by cell fusion of anaplastic thyroid carcinoma cell lines, which show a highly malignant phenotype, to cell lines deriving from differentiated thyroid carcinoma, which show a non-tumorigenic or a poorly tumorigenic phenotype. All of the parental cell lines showed impaired p53 gene function.

Results: The cell hybrids contained alleles from the parental cell lines. All of the cell hybrids showed a lower growth rate compared with the parental undifferentiated carcinoma cell lines and were unable to grow in soft agar and to induce tumours after injection into athymic mice.

Conclusion: Taken together, these findings suggest that the highly malignant phenotype of the anaplastic thyroid carcinoma is achieved by the impairment of gene functions that negatively regulate cell growth, rather than by the activation of dominant oncogenes.

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Introduction

Tumours of the thyroid gland are common in humans, even though only a minority of them are malignant. Thyroid carcinomas vary considerably in aggressiveness, ranging from the non-aggressive occult papillary carcinoma to the rapidly growing anaplastic carcinoma, the latter being one of the most lethal human neoplasms. Poorly differentiated and anaplastic carcinomas seem to be derived from the progression of papillary and follicular differentiated carcinomas (1, 2). This progression process is expected to require multiple changes in the expression of several genes. Some of these genes may be overexpressed in the anaplastic carcinoma compared with the differentiated tumours as demonstrated for *c-myc* (3), galectin (4, 5), β -10 thymosin (6) and *HIP* (7). However, it is also well known that conversion of a normal cell to a malignant one can result in the impairment of gene functions that negatively regulate cell growth (8). In fact, cell-fusion

experiments implied the existence of genes provided by the non-tumorigenic cells capable of suppressing tumorigenicity (9–13). These genes are also known as tumour suppressor genes, and recessive mutations in both of the alleles are required to give the cell a growth advantage. The aim of the present work was to determine the recessive or dominant phenotype of anaplastic thyroid carcinomas through cell fusion between differentiated and anaplastic thyroid carcinoma cell lines. Therefore, cell hybrids were generated between two anaplastic thyroid carcinoma cell lines, named ARO and FRO, and two papillary (NPA and NIM1) and one follicular (WRO) carcinoma cell lines. Interestingly, NIM1 and NPA cells are derived from a differentiated and a poorly differentiated papillary carcinoma respectively. This may account for the poorly tumorigenic phenotype of the NPA cells. In contrast, FRO and ARO cells are highly neoplastic. The NIM1 and WRO cell lines are completely non-tumorigenic (3, 14–17).

Here we report that the cell hybrids derived from the fusion of thyroid anaplastic and papillary or follicular carcinoma cell lines did not show the malignant phenotype, being unable to grow in agar and unable to induce tumours in athymic mice. These results suggest that the progression from differentiated to anaplastic thyroid carcinoma requires the loss of function of genes that negatively regulate thyroid cell proliferation.

Materials and methods

Cell culture

The human thyroid carcinoma cell lines used in these studies were WRO (15), NPA (16), ARO (3, 14), FRO (14) and NIM1 (17). They were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Transfections and plasmids

Thyroid carcinoma cell lines were transfected with the plasmids pSVneo and pSVhygro, which carry the genes for resistance to geneticin and hygromycin respectively, under the transcriptional control of the simian virus 40 (SV40) large T promoter. Transfections were performed with the calcium phosphate procedure as described (18).

Cell fusion

Cells were fused according to a published procedure (19). A mixture of 1×10^4 cells from both parental cell lines was seeded in a 60 mm dish overnight in growth medium supplemented with 10% calf serum. Cells were rinsed twice in phosphate-buffered saline and then with 4.0 ml 48% (w/v) polyethylene glycol (PEG) (M_r 1000) in serum-free medium for 45 s. The PEG solution was removed and the cells were rinsed three times with growth medium, and then incubated overnight. The following day, the cells were transferred from the 60 mm dish to six 100 mm dishes. Selective medium containing geneticin and hygromycin was added 2 days later.

Assay of the transformed state

The tumorigenicity of the cell lines was tested by injecting 2×10^6 cells subcutaneously into athymic mice. The sites of injection were observed at regular intervals for the appearance of tumour formation and progression. Cell lines were considered non-tumorigenic when no evidence of tumour formation was seen by 6 months after injection. Soft-agar colony assays were performed as described elsewhere (20).

Chromosomal analyses

Chromosomal studies were performed using standard methods (21). Metaphases were stained with Giemsa

and counted at low power for ploidy determination. The average number of chromosomes was determined from a minimum of 10 good metaphases.

Chromosomal analysis of parental and hybrid cell lines

Genomic DNA was isolated from both parental and hybrid cell lines by using a standard procedure (22). The DNA was used in PCR reactions with eight chromosome-specific fluorescent primers obtained from Research Genetics (Huntsville, AL, USA). Primer specificities were as follows: D1S1612 and D1S1609, chromosome 1; D3S2406, chromosome 3; D4S2394, chromosome 4; D6S1031, chromosome 6; D11S1986, chromosome 11; D12S1045, chromosome 12; GATA188F04, chromosome 21; D22S683, chromosome 22. Each 10 μ l PCR reaction tube contained 10 ng genomic parental or hybrid DNA, 1.5 mM *Taq* Gold Buffer (Perkin-Elmer, Branchburg, NJ, USA), 250 mM of each dNTP, 200 mM of each ABI PRISM fluorescent primer and 0.5 U *Taq* Gold Polymerase (Perkin-Elmer). After the activation of *Taq* Gold Polymerase at 95 °C for 15 min, 35 cycles of PCR were performed with a Perkin-Elmer thermal cycler 9700 at 95 °C for 30 s (denaturation), at various temperatures for 30 s (annealing) and at 72 °C for 30 s (extension). PCR reaction samples were mixed with sequencing gel loading solution, heated at 95 °C for 2 min and separated by electrophoresis on 5% denaturing polyacrylamide gels (8 M urea) by using an automatic sequencer (ABI PRISM 377, Applied Biosystems, Foster City, CA, USA) using a CEPH (Cooperative Human Linkage Center, Research Genetics, Huntsville, AL, USA) pedigree members 1331-01 and 1331-02 as standards.

RNA isolation and Northern blot analysis

RNA was extracted from the parental and hybrid cells by using previously described procedures (22). Northern blots and hybridizations have been described previously (3). The *c-myc* probe used in this study was the 1.3 kb PstI-PstI fragment of the pRyc 7.4 plasmid, which is specific for the human *myc* gene (3). A mouse β -actin probe was used to ascertain equal RNA loading (3).

Results

Features of the parental thyroid carcinoma cell lines and production of thyroid carcinoma cell hybrids

The characteristics of the parental thyroid carcinoma cell lines are summarized in Table 1. ARO and FRO are highly tumorigenic: in fact, they grow with good efficiency in soft agar and are able to induce invasive tumours with a short latency period in athymic mice.

Table 1 Human thyroid carcinoma cell lines and fusion combinations for generation of hybrid cell lines.

Cell lines	Histotype	Chromosomes (average)	Chromosomes (range)	Doubling time (h)	Agar growth	Tumour incidence ^a	Latency period ^b (weeks)
Parental line							
ARO	Anaplastic	65	60–71	6	+++	5/5	2–3
FRO	Anaplastic	75	68–79	10	++	5/5	2–3
NIM	Papillary	92	87–96	16	–	0/5	–
NPA	Papillary ^c	57	50–61	24	+	3/5	8–10
WRO	Follicular	81	77–88	24	–	0/5	–
Hybrid line							
ARO–ARO	/	–	–	6	+++	5/5	2–3
FRO–FRO	/	109	101–119	10	++	5/5	2–3
WRO–NPA	/	137	130–143	19	–	0/5	–
WRO–ARO	/	127	122–134	16	–	0/5	–
WRO–FRO	/	97	90–104	28	–	0/5	–
FRO–NIM	/	109	99–112	15	–	0/5	–

^a Subconfluent dishes were trypsinized, centrifuged and resuspended in serum-free media to 10^7 cells/ml. Cells (5×10^6) were injected s.c. under each haunch of nu/nu mouse. Incidence is represented by the number of tumours that developed relative to total sites injected. Complete absence of tumour formation was scored after 200 days.

^b The latency period is the time from injection until the tumour was first palpable.

^c Poorly papillary differentiated carcinoma.

Conversely, WRO and NIM1 are not able to grow in agar and are not tumorigenic in athymic mice. The NPA cells present intermediate behaviour, showing the ability to grow in a semi-solid medium, albeit at low efficiency, and to give rise to small tumours (3). To generate cell hybrids between carcinoma thyroid cell lines, each cell line was transfected with the plasmids pSVneo or pSVhygro, which carry the genes for resistance to geneticin and hygromycin respectively. The cells were selected in media containing the selecting drug. Several clones and the pool population were picked up for each transfection. Subsequently, fusions were performed between different cell lines selecting the hybrid cells in a medium containing both geneticin and hygromycin. In this case also, several clones and the pool populations were picked up. Three clones and the pool population were further analysed for growth rate, growth in soft agar and tumorigenicity. The results presented here refer to one representative cell clone; however, similar results were obtained with all of the clones derived from the same cell fusion. The five cell lines and the four fusion combinations are described in Table 1.

Cytogenetic characterization

All of the hybrid lines were examined for their chromosomal composition to confirm that they were true hybrids. The chromosomal features of both hybrids and parental lines are summarized in Table 1. These results provide firm evidence that the cell lines were really hybrids. In fact, they always contained more chromosomes of the parental cell lines. The parental tumour lines are mostly hypotriploid, whereas the hybrid cell lines are mostly hypotetraploid or hypopentaploid. In some cases, the number of chromosomes in the hybrids corresponded to the sum of the chromosome

numbers of the parental cell lines (see the WRO–NPA cell hybrid).

Moreover, to obtain further verification that the cell lines recovered after fusion and selection in G418-hygromycin medium were actually hybrids of the parental cell lines, we used pairs of PCR primers that span highly polymorphic sequences on specific chromosomes (23, 24). Primer pairs were first screened to identify those that yielded size polymorphisms in the different cell lines; then genomic DNA, isolated from each of the hybrid cell lines and from the parental cells, was amplified by PCR with these pairs of primers. The analysis of PCR products obtained with eight primer pairs representing different chromosome microsatellites is summarized in Table 2. Entries in Table 2 show the sizes (in nucleotides) of the products obtained, indicating which alleles are present. They indicated that the hybrids contained alleles from both parental cell lines.

Therefore, although not all of the chromosomes were analysed, the results demonstrate that the selected cell lines represent true hybrids between the two parental cell lines.

Morphology and growth rate of the cell hybrids

In Fig. 1, we show the morphology of the parental cell lines and the cell hybrids. Generally, the morphology of the cell hybrids was different from that of both parental cell lines. The hybrids generated from the ARO cell line lost the round shape typical of the ARO cells and showed greater adhesion to the plate, suggesting a reduced malignant phenotype.

The growth rate of the hybrid lines was determined (see the results reported in Table 1). The hybrid cells showed a growth-rate reduction that is particularly

Table 2 Analysis of selected chromosomes of parental and hybrid cell lines.

Marker	Product size (nucleotides) ^b								
	ARO	WRO	NIM	NPA	FRO	WRO-NPA	WRO-ARO	WRO-FRO	FRO-NIM
1 (D1S1612)	110,122	118	96,122	96,126	118,126	96,118,126	118,122,110	118,126	118,122,126
1 (D1S1609)	180,200	204	204	200	184,200	200,204	180,204,200	184,200	184,200,204
3 (D3S2406)	334,338	334,346	338,354	334,350	322,350	334,346,350	338,346	334,346,350	322,354,338
4 (D4S2394)	238	238,250	241	256	241	238,250,256	238,250	238,241,250	241
6 (D6S1031)	248,264	264	248,261	248,264	248,258	248,264	264,248	248,258,264	248,261
11 (D11S1986)	196,228	192,240	196,204	192,248	232,240	192,240,248	192,196,228,240	192,232,240	196,204,232,240
12 (D12S1045)	76,80	92	94,76	88,92	92,94	88,92	76,92	92,94	92,94,76
21 (GATA188F04)	125,131	193	117,193	117,157	129,157	117,157,193	193,131	129,157,193	129,157,193
22 (D22S683)	176,204	180	168,172	168,172	176	168,172,180	180,204	176,180	168,176

^a Markers were primer pairs (identified in parentheses) that are specific for each of the indicated chromosomes.

^b Chromosomal DNA was isolated from each of the indicated parental and hybrid cell lines, and PCR reactions were carried out using the indicated primer pairs. Reaction products were separated on DNA sequencing gels. Entries in the table show the size (in nucleotides) of the products obtained, indicating which alleles were present.

striking for the FRO-WRO hybrid, which showed a growth rate (28 h) even lower than that of the WRO cells (24 h). No changes in the cell growth and morphology of the parental-parental self-hybrids (i.e. FRO-FRO, ARO-ARO and NPA-NPA) in comparison to the respective parental cell lines were observed (data not shown), thus excluding the possibility that the slower growth of hybrid cells was solely dependent on the fusion process.

Analysis of the malignant phenotype of hybrids

All the hybrid cells were found to be unable to grow efficiently in soft agar and to be unable to give rise to tumours when injected into athymic mice (Table 1). Conversely, the FRO-FRO and ARO-ARO cells grew efficiently in soft agar and were highly tumorigenic in athymic mice.

Therefore, the fusion of the anaplastic thyroid cell lines with papillary and follicular cell lines results in the suppression of the malignant phenotype. Interestingly, even the fusion of the poorly tumorigenic and poorly differentiated carcinoma NPA cells with the follicular carcinoma cell line WRO gives rise to a hybrid cell line that is unable to grow in soft agar, unable to induce tumours in athymic mice and which has a reduced growth rate relative to the parental cell lines; this indicates that a tumour suppressor gene may be involved in the progression step driving the cells from a differentiated carcinoma to a poorly differentiated papillary carcinoma. We also evaluated the expression of genes, such as *c-myc*, correlated with the aggressive phenotype (3) in hybrid cells: they were expressed at lower levels compared with the parental anaplastic cell lines (data not shown).

Discussion

Malignant tumours of the thyroid gland include differentiated and undifferentiated carcinomas. The

latter are probably derived from the progression of the former ones (1, 2). It can be assumed that the anaplastic phenotype of thyroid tumours may be achieved through the acquisition of the ability to express genes involved in tissue invasion or cell proliferation and/or the inactivation or deletion of tumour suppressor genes. Alterations in the *p53* tumour suppressor gene have been described as a peculiar feature of the anaplastic carcinomas (25). However, from the data present in the literature, deletion or mutations of the *p53* gene do not seem to be the only alteration present in anaplastic carcinomas. In fact, thyroid tumours do not appear in Li-Fraumeni patients and *p53* null mice, suggesting that other genes (26, 27) (oncogenes or suppressor genes) may be involved in the process of carcinogenesis leading to the anaplastic phenotype. To date, the oncogenes that have been found activated in human thyroid tumours, such as *RET/PTC* and *TRK*, have never been found associated with *p53* mutations (25) and *Ras* mutations have been found only in two cases associated with mutations in the *p53* gene (28).

Even *in vitro* co-operation between activated *ras* and mutated *p53* genes does not lead to the achievement of the neoplastic phenotype in thyroid cells (F. Trapasso and A. Fusco, manuscript in preparation). Moreover, loss of heterozygosity on chromosome 10q22 has been observed in two follicular carcinomas adjacent to an area of anaplastic carcinomas (29, 30). These data would support the involvement of other suppressor genes in the progression process leading to the anaplastic phenotype. To validate this hypothesis, hybrid cell lines were generated by the fusion of cell lines originating from thyroid undifferentiated carcinomas with those originating from differentiated carcinomas, including the papillary and the follicular histotype. Cytogenetic studies of the hybrid lines confirm that they were true hybrids. In fact, they contain more chromosomes than the parental cell lines (in some cases, the chromosome number was close to

Figure 1 Phase-contrast photomicrographs of parental and hybrid cell lines. Magnification: 150 $\times$.

the sum of those of the fusion partners). Moreover, the analysis of polymorphic sites showed that the hybrids retained the alleles of both parental cells.

The results reported here provide evidence that the loss of some genes is required for progression towards the anaplastic thyroid phenotype. In fact, the hybrids showed the abolition of the markers of malignancy, such as growth in agar and tumorigenicity in athymic mice. Furthermore, these hybrids were less spindle-shaped, there was an increase in adhesiveness to cell plates and there was a reduction in the growth rate. In this report, we also show that the hybrid NPA–WRO is not tumorigenic: this might indicate that the poorly tumorigenic phenotype of a poorly differentiated papillary carcinoma might be correlated with the loss of some tumour suppressor activity.

The possibility of the involvement of the *p53* wild type in the suppression of the malignant phenotype of the hybrids can be excluded since all of the parental cell lines show impaired *p53* gene function (14), which represents a feature of all the established thyroid carcinoma cell lines that is independent of histotype. In fact, ARO, WRO and NPA showed mutations in the *p53* gene at codons 273, 223 and 266 respectively, whereas the FRO cell line showed a drastic reduction in *p53*-specific mRNA expression (14). Moreover, analysis of the hybrids revealed the presence of the mutated *p53* proteins (data not shown). Therefore, changes in the expression of the malignant phenotype by the cell hybrids can be considered to be independent of the presence of a wild-type *p53* protein.

In conclusion, the data reported here imply the presence in papillary and follicular carcinoma cells of genes able to block the aggressiveness of the anaplastic thyroid carcinoma cell lines, suggesting that the same genes were somehow inactivated in the original tumour. Moreover, the cell hybrids reported here may represent an excellent cell-based system for the identification of genes (probably tumour suppressor genes) involved in human thyroid carcinogenesis.

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