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Oncology in Focus

Amplification of the Int-2 gene in head and neck squamous cell carcinoma

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Abstract

Cellular oncogenes have been implicated in head and neck cancer development since 1986. More recently interest has focused on chromosome 11q13; oncogenes therein undergoing ongoing investigation include Bcl-1/Prad-1, Hst-1 and Int-2.

Our laboratory has studied the Int-2 oncogene for several years, primarily in the breast. This paper presents our investigations of Int-2 in the head and neck. Thirty-four paraffin-embedded primary squamous cell carcinomas were studied for Int-2 gene amplification using a carefully controlled method of sequence quantification by DNA dot blots. Amplification, mostly low level, was identified in 62 per cent of samples studied. No clinical correlation to amplification could be found.

Further studies are underway looking for evidence of expression of Int-2 in fresh tissues and for amplification and expression of other oncogenes on this amplicon.

Key words: Head and neck neoplasms; Carcinoma, squamous cell; Gene amplification

Introduction

Cellular oncogenes have been found to be involved in the control of normal cell growth and development. Research over the past decade has indicated that abnormal activation of these oncogenes may result in the expression of the malignant phenotype of cancer cells (Bishop, 1987). It is only recently, however, that oncogenes have been implicated in head and neck cancer, the first reports coming out in 1986 (Yokota *et al.*, 1986). Since then, numerous studies of squamous cell carcinomas of the head and neck have implicated cellular oncogene involvement (Field *et al.*, 1986; Field and Spandidos, 1987; Hoellering and Shuler 1989).

Considerable data from these studies have suggested that genes found in the amplicon on chromosome 11q13 might be involved in squamous cell carcinoma of the head and neck. Amplification has been identified in the genes which are present in the 11q13 amplicon; these include Bcl-1/Prad-1 (Berenson *et al.*, 1989; Somers and Schechter, 1992), Hst-1 (Tsuda *et al.*, 1989), and Int-2 (Zhou *et al.*, 1988; Merritt *et al.*, 1990; Somers *et al.*, 1990; Somers and Schechter, 1992).

Our laboratory has studied the oncogenic potential of the Int-2 gene for several years. Initial work involved the study of the development of mammary tumours in the mouse as a result of infection with the mouse mammary tumour virus (MMTV). The Int family of proto-oncogenes were then found to be activated by MMTV infection (Dickson *et al.*, 1984; Casey *et al.*, 1986; Gallahan and Gallahan, 1987; Zhou *et al.*, 1988) and our work has focused on the role of these genes in mouse breast tumour development (Etkind, 1989). We are also involved in the study of the Int-2 gene in human breast tumour development since approximately 18 per cent of human breast tumours show Int-2 gene amplification (personal communication, P. Etkind).

More recently we have been investigating the role of Int-2 in head and neck squamous cell carcinoma (HNSCC). Using a carefully controlled method of sequence quantification by DNA dot blots (Heerdt *et al.*, 1991), we have been able to identify low level amplification of DNA in paraffin-embedded sections. To date we have investigated Int-2 amplification in 34 patients with head and neck squamous cell carcinomas.

Materials and methods

DNA was isolated from paraffin-embedded samples of 34 patients with squamous cell carcinoma of the head and neck. Both tumours and normal tissues were evaluated.

DNA isolation

DNA was isolated from paraffin sections from which 150 5 μm slices had been shaved. These slices were placed in a 2.2 ml Eppendorf tube (usually two tubes for 150 slices), to which was added 462.5 μl Tris-EDTA, pH 9.0, 25 μl 20 per cent SOS and 12.5 μl proteinase K (20 mg/ml). These samples were incubated at 48°C for 72 hours with shaking. At both 24 and 48 hours an additional 12.5 μl proteinase K (20 mg/ml) were

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added. At 72 hours samples were extracted with 500 µl phenol and 500 µl chloroform: isoamyl alcohol (99:1). To the final volume of the aqueous phase 1/10 volume 2.5 M ammonium acetate and 2.5 volumes of cold ethanol were added. Samples were allowed to precipitate overnight at −20°C. Samples were brought to room temperature, spun for 30 minutes in a microfuge, the supernatant was decanted, and cold 70 per cent ethanol was added to rinse the pellets. Samples were then spun for 15 minutes, decanted, and the pellets were air-dried. Pellets were resuspended in 10 mM Tris EDTA, pH 8, to a final volume of 100 µl (Goelz *et al.*, 1985). DNA concentration from paraffin sections was determined by optical density readings.

Detection of DNA amplification

Amplification of the Int-2 gene was analysed by the dot blot procedure (Thomas, 1980) with stringent quantification (Heerdt *et al.*, 1991) to allow us to detect even low levels of amplification. All samples were done in triplicate with each prepared DNA sample being divided in half before being dotted. All blots also contained placental DNA as internal controls. Blots were hybridized to either the Int-2 probe or to the beta-globin gene probe as a single copy gene probe.

After restriction enzyme digestion and consequent boiling for five minutes to denature the DNA, 40 µl samples containing 50 µg DNA were brought to a final volume of 200 µl with the addition of 1 M ammonium acetate. Nitrocellulose paper used for dot blotting was soaked in distilled water for 10 minutes. The dot blot apparatus was assembled using one piece of gel blot filter paper wet in 1 M ammonium acetate under the nitrocellulose. Dot blot wells were rinsed with ammonium acetate and each duplicate well received 97 µl of each sample. Wells were rinsed with 1 M ammonium acetate. The apparatus was disassembled and the DNA dotted onto the nitrocellulose was covalently bound to the nitrocellulose in a UV Stratalinker. The nitrocellulose was air-dried and prehybridized for two to four hours at 65°C with shaking in hybridization buffer containing 5 × SSC (20 × SSC = 3M NaCl, 0.3 M Na citrate), 5 × Denhardt's solution (Denhardt, 1966), 100 µg/ml salmon sperm DNA, and 0.5 per cent SDS (sodium dodecylisulphate). The human Int-2 probes which we used were supplied by Dr Clive Dickson from the Imperial Cancer Research Fund Laboratories in London, England. The beta-globin probe was used as a single copy gene control. The probes were radioactively labelled to a specific activity of 10^9 cpm/µg DNA by the random priming procedure using p32 labelled-dCTP and dATP. Probes were denatured by boiling for five minutes and immediately added to fresh hybridization buffer. Dot blots were hybridized for 24–48 hours at 65°C with shaking. Probes were removed and the blot was washed by shaking for 15 minutes at room temperature in 250 ml of 2 × SSC/0.5 per cent SDS and 4 × for 15 minutes at 65°C in 250 ml of 1 × SSC/0.1 per cent SDS, air-dried, and put on photographic film.

Quantification of DNA amplification

The procedure that we found to be most accurate for determining gene amplification involved cutting out each dot after high stringency hybridization from hybridized nitrocellulose paper, placing each cut dot into a separate scintillation vial, and counting each sample in a liquid scintillation counter. Standardized levels of hybridization relative to the placenta were determined as follows:

$$\text{Sample ratio} = \frac{\text{Int-2 counts/sample}}{\text{Beta-globin counts/duplicate sample}}$$

$$\text{Average placental ratio} = \frac{\text{Int-2 count/placental sample}}{\text{Beta-globin counts/placental sample}}$$

$$\text{Level of gene amplification} = \frac{\text{Sample ratio}}{\text{Average placental ratio}}$$

As described by Heerdt *et al.* (1991), there are several noteworthy features of sequence quantification using this method. These features include: (1) multiple determinations made on each DNA sample; (2) small variations in concentration among DNA samples normalized by beta-globin hybridization; (3) copy numbers are expressed relative to the same standard placental DNA sample present in each experiment; and (4) in contrast to scanning densitometry, fewer variables (such as sample transfer, variations in background and grains on the film and linearity of film exposure, particularly with intensifying screens) are introduced into this dot blot method.

Biostatistics

Biostatistical analysis was performed at the Biostatics Department of Montefiore Medical Centre. It was performed in the following ways:

(1) Descriptive statistics were obtained.

(2) Amplification was used as a continuous variable and scatterplots were run for T-stage, nodal status and differentiation; a Spearman-Rank correlation was performed for above.

(3) Two dichotomous amplification variables were created ($\geq$1.5 amplification; $\geq$2.0 amplification); dichotomous variables for T-stage, nodal status and differentiation were created. Using the Fisher's exact test, these variables were compared.

(4) Using dichotomous variables of amplitudes, T-staging, nodal status, differentiation for strata, the proc life test was performed. It was also performed using continuous amplifications as the variable.

Results

Int-2 copy number in head and neck squamous cell carcinomas was determined using a carefully controlled method of sequence quantification by DNA dot blots (Heerdt *et al.*, 1991). This method is capable of detecting small differences in gene copy number which may not be obvious by standard Southern blot methodology. Thirty-four primary squamous cell carcinomas from various sites of origin in the head and neck from 28 males and 6 females with a median age of 61 were analysed. Results of stringent dot blot hybridizations revealed the presence of low levels of Int-2 gene amplification in 62 per cent of the samples studied. Normal tissue showed no amplification.

As seen in Figure 1, 12 per cent (four tumours) demonstrated >3-fold amplification (two tumours 3.0 to 3.5-fold, one tumour 3.5 to 4.0-fold and one tumour 4.5 to 5.0-fold), 18 per cent (six tumours) demonstrated 2.5 to 3.0-fold amplification, six per cent (two tumours) demonstrated 2 to 2.5-fold amplification, 26 per cent (nine tumours) demonstrated 1.5 to 2.0-fold amplification and 38 per cent (13 tumours) demonstrated less than 1.5-fold amplification.

Using amplification as the independent variable, and evaluating for T-stage, nodal status and differentiation, no statistical significance was identified in these specimens. This was the case if amplification of $\geq$1.5 was used or if amplification of $\geq$2.0 was used as representing significant level of amplification (Table I).

There was a minor trend toward increased likelihood of amplification for advanced T stage ($T_{3,4}$) in patients with $\geq$2.0 amplification (67 per cent $T_{3,4}$ *versus* 42 per cent $T_{1,2}$) (Table I). This was not statistically significant, and only had a power level of 0.27. It is estimated that 126 patients would need to be studied for it to have a power function of 80 per cent, and be deemed significant.

Reviewing survival statistics with relation to amplification, no statistical significance could be drawn from this data. Survival curves were similar. Of interest, in this limited sample, no survival differences were reflected by T-stage or by differentiation.

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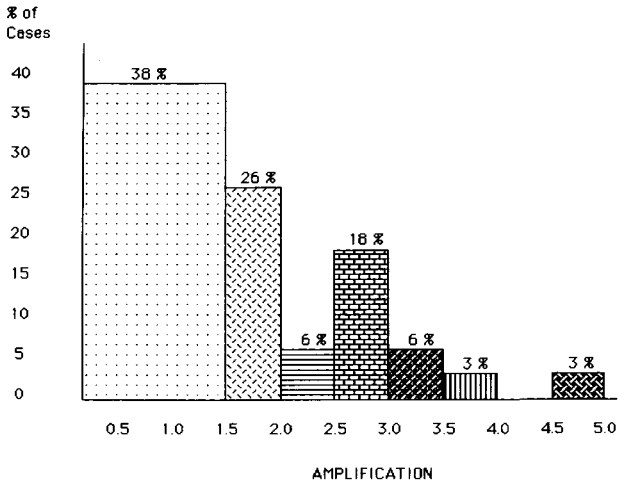

FIG. 1
Amplification data of Int-2.

Only the presence of nodal disease could be identified as statistically making a difference in survival ($p = 0.0001$) (Table I).

Discussion

Neither the cause nor the mechanism of gene amplification in mammalian cells is known, but the abnormality has generally been found in cells that are partially or fully malignant. A large number of tumours and tumour cell lines have been screened for the amplification or overexpression of known proto-oncogenes. The screening has uncovered a variety of tumours in which one or other proto-oncogene is expressed abundantly because it resides within an amplified domain of DNA.

Proto-oncogenes recognized as consistent components of amplified DNA include *myc* in carcinomas of the breast (Escot *et al.*, 1986) and lung (Little *et al.*, 1983), the closely linked Hst-1 and Int-2 in carcinoma of the breast (Zhou *et al.*, 1988); *neu* in carcinoma of the breast and other adenocarcinomas (Van de Vijver *et al.*, 1987); N-*myc* in neuroblastoma (Schwab *et al.*, 1983); N-*myc* and 1- *myc* in small cell carcinoma of the lung (Nau *et al.*, 1985).

Carcinogensis of the head and neck is a multistep process. Numerous studies of HNSCC have implicated cellular oncogene involvement (Field *et al.*, 1986; Field and Spandidos, 1987; Hoellering and Shuler, 1989; Berenson *et al.*, 1989). The association between the exposure of tissues of the upper aerodigestive tract to tobacco and alcohol and the incidence of head and neck squamous cell carcinoma is consistent with current animal models of carinogenic exposure and oncogene amplification (Wong and Biswas, 1987). Chromosome instability persisting over periods of time and affecting a high percentage of dividing cells is likely to increase the risk of malignancy. The oral mucosa of smokeless tobacco users appears to represent such a case (Stich *et al.*, 1991) as does that of smokers. The relative risk of development of oral cavity carcinoma in smokers with known

TABLE I
RESULTS

Name	Age (years)	Sex	Tumour site	Stage	Differentiation	Amplification	Comments
CM	60	M	FOM	$T_4N_1M_0$	W	4.77	Dead NED
EL	73	M	Pyriform	$T_4N_1M_0$	P	3.76	DOD
TC	64	M	FOM	$T_3N_0M_0$	M	3.13 (1.13)	Dead NED
JS	51	F	FOM	$T_1N_0M_0$	W	3.06	DOD
WT	76	M	Pyriform	$T_4N_0M_0$	M	2.96 (2.75)	DOD
AQ	69	F	Alveolus	$T_2N_0M_0$	W	2.87	NED
GK	84	M	Hypopharynx	$T_3N_0M_0$	M-P	2.76	NED
WH	78	M	Pyriform	$T_1N_0M_0$	M	2.62	Dead NED
VA	57	M	Tonsil	$T_3N_2M_0$	M	2.57 (1.93) (1.55)	DOD
MV	73	M	Pharynx	$T_4N_3M_0$	P	2.57	DOD
JM	61	F	Tongue, FOM	$T_3N_1M_0$	M	2.35 (1.10)	DOD
JC	53	M	FOM	$T_2N_1M_0$	W	2.34 (L.N. 1.06)	DOD
CH	53	M	FOM	$T_1N_0M_0$	W	1.98 (1.95)	NED
WF	53	M	FOM	$T_1N_0M_0$	M	1.96	NED
RH	84	M	Hypopharynx	$T_2N_0M_0$	W	1.94	Dead NED
FM	65	M	Tonsil	$T_3N_3M_1$	W	1.83	DOD
PC	49	M	FOM	$T_4N_0M_0$	W	1.77	NED
CW	56	M	FOM	$T_2N_0M_0$	W	1.74	Dead NED
LG	84	M	FOM	*	M	1.72	
SH	51	F	BOT	$T_3N_2M_0$	M-P	1.67	DOD
RA	61	M	Tongue	$T_3N_2M_1$	W	1.56	DOD
HK	55	M	BOT	$T_3N_2M_0$	W	1.49 (1.26)	DOD
SG	46	F	Cheek	$T_1N_1M_0$	M	1.44	DOD
CW	71	M	Pyriform	$T_1N_2M_0$	*	1.28	NED
TK	69	M	RMT	$T_3N_2M_0$	M	0.98	DOD
PJ	*	M	FOM		W	0.98	
FM	72	M	Larynx	$T_2N_0M_0$	P	0.93	NED
TF	80	M	Parotid	$T_2N_1M_0$	*	0.89	DOD
GN	56	M	FOM	$T_4N_2M_0$	W (nodes P)	0.86	DOD
CC	56	M	Pharynx	$T_3N_0M_0$	W-M	0.78	NED
LF	67	M	FOM	$T_2N_0M_0$	W	0.72	NED
EA	61	F	Larynx	$T_3N_0M_0$	M-W	0.69	NED
RN	51	M	FOM	$T_4N_0M_0$	W	0.66	NED
HS	48	M	Pyriform	$T_4N_1M_0$	*	0.60	DOD

*Data not available.
Key: FOM = floor of mouth; RMT = retromolar trigone; BOT = base of tongue; W = well differentiated; P = poorly differentiated; M = moderately differentiated; M–P = moderately to poorly differentiated; W–M = well to moderately differentiated; M–W = moderately to well differentiated; NED = no evidence of disease; DOD = dead of disease.

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chromosomal instability is 19.8 (Sloan and Goepfert, 1991). Recently it has been shown that the frequency of fragile sites is markedly higher in peripheral blood lymphocytes in smokers compared to those in nonsmokers especially at five chromosomal locations (Kao-Shan *et al.*, 1987). One of the specific sites identified was at 11q13.

Amplification of band q13 of chromosome 11 is a consistent feature in breast cancer and head and neck squamous cell carcinoma. This has led several groups of investigators to focus their investigations of oncogenes which may be important in the genesis of head and neck squamous cell carcinoma to this amplicon.

There are three proto-oncogenes actively under current investigation from this amplicon; these include Int-2, Hst-1 and Bcl-1/Prad-1.

We have been interested in investigating the Int-2 gene and HNSCC. Int-2 (FGF-3), like Hst-1, is a member of the family of FGF-related genes, with 44 per cent amino acid homology to basic FGF (Casey *et al.*, 1986; Somers *et al.*, 1990).

Int-2 appears to be produced in precise steps of embyro development but has not been detected in any normal adult tissue (Wilkinson *et al.*, 1989). Int-2 RNA is present in cells of the primitive streak. In the mouse embryo, Int-2 transcripts have been detected in the rhombencephalon at a developmental stage consistent with induction of the inner ear; in fact Int-2 appears to constitute a signal for induction of the otic vesicle (Represa *et al.*, 1991).

In the mouse embryo at day 8.5 to 9.5, one of the main sites of expression of the Int-2 gene is in the pharyngeal pouches (Wilkinson *et al.*, 1989), which in the primitive foregut elongate to form portions of the developing head and neck; thus it is of particular interest as a proto-oncogene in HNSCC.

The first report linking Int-2 with HNSCC was by Zhou *et al.* (1988) who noted amplification in two out of eight HNSCC. Others who have studied it more recently include Somers *et al.* (1990) and Merritt *et al.* (1990) each of whom found amplification in respectively, 10 out of 21 and 11 out of 21 HNSCC.

Our data represent a larger series then either Somers' or Merritt's, but roughly parallels their findings. Of our 34 tumours studied, 62 per cent or 21/34 demonstrated amplifications of Int-2 of 1.5 times normal or greater. Our techniques allowed for identification of low level amplification; nine patients in this group demonstrated amplification of 1.5 to 2 times normal. Due to this capacity we were able to identify a slightly higher percentage of amplification than the other groups. We did not identify any clinical significance in the group which had significant amplification, however.

It is necessary to determine if Int-2 is expressed in head and neck squamous cell carcinoma. In general, there has not been clear cut evidence of its expression at the RNA level (Basilico and Moscatelli, 1992). The first few fresh samples of HNSCC which we have examined have not demonstrated expression. The general explanation for this lack of expression is either that Int-2 may be a passive bystander on chromosome 11q13, or the amplification of Int-2 represents a previous event in the evolution of the tumours studied and is no longer essential for tumour growth (Basilico and Moscatelli, 1992). We will continue our investigations of Int-2 in paraffin-embedded and fresh tissue from HNSCC, as well as also investigating other oncogenes on the 11q13 amplicon, in particular Bcl-1/Prad-1.

Conclusion

Thirty-four cases of primary squamous cell carcinoma of the head and neck were investigated for amplification of the proto-oncogene Int-2 located on chromosome 11q13 using a carefully controlled method of sequence quantification by DNA dot blot. Amplification of the Int-2 gene was identified in 62 per cent of specimens (21/34) studied. Amplification was low level, the largest group had amplification in the 1.5 to 2.0 times range. This data is consistent with that of other investigators.

Further work will involve investigation of other genes on the same amplicon as well as investigation of amplification and expression of the Int-2 gene in fresh head and neck tumour samples.

Glossary

Amplicon.
: Region on a chromosome containing increased copies of genes.

Bcl-1/Prad-1 gene.
: A gene present on the q13 region of chromosome 11 and often amplified in a number of tumour types; Bcl-1 and Prad-1 have recently been shown to be the same gene and to code for a cytoplasmic protein called cyclin D1 which regulates cell cycle progression from G1 to S phase.

Chromosome 11q13.
: Region on the long arm of chromosome 11 which is often amplified in a number of tumour types.

Denature.
: Separate DNA into its respective two strands.

DNA dot blots.
: Nitrocellulose paper containing individual DNA samples dotted onto the paper.

Gene amplification.
: Increased copy number of a specific gene.

Gene expression.
: Copying of gene into messenger RNA.

High stringency.
: Hybridization conditions in which only totally complementary nucleic acids can bind together.

Hst-1 gene.
: A gene present on the q13 region of chromosome 11 and often amplified in a number of tumour types; Hst-1 is a fibroblastic growth factor related gene.

Hybridize.
: Allowing complementary strands of nuclei acid to bind together.

Int-2 gene.
: A gene present on the q13 region of chromosome 11 and often amplified in a number of tumour types; Int-2 is a fibroblastic growth factor related gene.

Oncogene.
: Gene that can induce one or more characteristics of neoplastic transformation.

Overexpression.
: Excess number of messenger RNAs produced from a specific gene.

Proto-oncogenes.
: Normal cellular genes that can be converted to active oncogenes by mutation or overexpression.

Restriction enzyme digestion.
: Cutting of DNA into size fragments with enzymes which recognize and cut at specific nucleotide sequences.

Transcripts.
: Messenger RNAs.

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