TP53 mutations, amplification of P63 and expression of cell cycle proteins in squamous cell carcinoma of the oesophagus from a low incidence area in Western Europe

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Summary In Europe, high incidence rates of oesophageal squamous cell carcinoma (SCCE) are observed in western France (Normandy and Brittany) and in north-eastern Italy. Analysis of TP53 mutations in tumours from these regions has shown a high prevalence of mutations at A:T basepairs that may result from DNA damage caused by specific mutagens. However, the spectrum of TP53 mutations in regions of low incidence is unknown. We report here TP53 mutation analysis in 33 SCCE collected in Lyon, an area of low incidence. These tumours were also examined for MDM2 and P63 amplification, and for expression of p16INK4a/CDKN2a, cyclin E, p27Kipl and Cox2. TP53 mutations were detected in 36% of the cases (12/33). In contrast with regions of high incidence, the mutation spectrum did not show a high prevalence of mutations at A:T base pairs. P63 was amplified in 5/32 cases tested (15.5%). No amplification of MDM2 was found. Expression studies revealed frequent loss of p16INK4a/CDKN2a (46%) and p27Kipl (25%) expression, and frequent overexpression of Cyclin E (70%) and Cox2 (42%). Overall, these results indicate that in Europe, SCCE from areas of high and low incidence present a similar pattern of molecular alterations but differ by the type of TP53 mutations. © 2001 Cancer Research Campaign

Keywords: oesophagus; squamous cell carcinoma; low-incidence area; TP53; P63; MDM2

Squamous cell carcinoma of the oesophagus (SCCE) is the sixth most frequent cancer worldwide (Pisani et al, 1999). Incidence rates vary greatly between different parts of the world (for review, see Muñoz and Day, 1996). Areas of extremely high incidence are found in northern Iran (Turkoman plain) and central China (Henan province and Shanxi), where incidence rates (ASR) of over 100/100 000/year have been reported in both men and women. Other, less clearly defined high incidence areas are found in parts of South America and in south and east Africa (Muñoz and Day, 1996). In Europe and in the United States, the overall incidence does not exceed 10/100 000/year in men and 2/100 000/year in women. However, in several areas, higher incidence rates have been observed exclusively in men (up to 30/100 000/year). These areas include the north-west of France (Normandy and Brittany) and the north-east of Italy (Veneto) (Launoy et al, 1994; Parkin et al, 1997; Zambon et al, 2000). In industrialised countries, it is estimated that 90% of SCCE is attributable to tobacco and alcohol consumption, with a multiplicative effect in individuals exposed to both factors (Tuyns, 1987). In the rest of the world, a variety of cultural and dietary habits have been incriminated. These include the consumption of scalding hot beverages and a deficiency in fresh fruits and vegetables (in most areas of very high incidence), a high dietary nitrosamine content (in China and south-east Asia) and the oral consumption of opium by-products (in Northern Iran) (Muñoz and Day, 1996).

Mutation of the TP53 gene is the most frequent genetic alteration described to date in SCCE, occurring in 35% to 70% of cancers, depending on the study and on the geographical origin of the tumours (Tanière et al, 2000b). Mutations are thought to occur at an early stage and have been observed in dysplasia, in normal mucosa adjacent to cancer lesions, and in oesophagitis in individuals from Normandy (Mandard et al, 2000). By comparing tumours from China, Thailand and western Europe, we have recently shown that the distribution and the nature of TP53 mutations varied according to their geographic origin. This raises the possibility that the mutation pattern may provide clues on the nature of the specific mutagens involved in oesophageal carcinogenesis (Tanière et al, 2000b).

The data available on TP53 mutations in tumours from Western Europe are essentially limited to areas of high incidence of Normandy, Brittany and north-eastern Italy (Hollstein et al, 1991; Audrezet et al, 1993; Esteve et al, 1993; Li et al, 2000; Robert et al, 2000; Shirvani et al, 2000). In these areas, many mutations occur at A:T basepairs (45% of all mutations), a type of mutation which is infrequent in other cancers and in SCCE from other parts of the world. This type of mutation is consistent with DNA damage inflicted by acetaldehyde, the first metabolite of ethanol (Tudek et al, 1999). Interestingly, several studies have shown an association between a functional polymorphism in aldehyde dehydrogenase 2 (ALDH2) and the risk of SCCE, suggesting that acetaldehyde may represent an important oesophageal carcinogen (Yang et al, 1999; Aggarwal et al, 2000).
In the present study, we have analysed TP53 mutations in a cohort of 33 SCCE patients from a low-incidence area of southeastern France (Lyon, Rhône-Alpes region). In this region the reported incidence of SCCE (ASR) is 10/100 000/year in men and 1/100 000/ in women (Parkin et al., 1997). We have also analysed amplification in genes suspected to play a role in the pathogenesis of SCCE, P63 and MDM2. P63 encodes a homologue of p53 that plays an essential role in the development of squamous epithelial. Mice lacking this gene die at birth from multiple defects due to improper skin formation (Yang et al., 1999). This gene is often amplified in primary human squamous carcinomas of the lung and the head and neck (Hibi et al., 2000; Yamaguchi et al., 2000). MDM2 encodes a protein that binds to p53, inhibits its transcriptional activity and induces its degradation. Several studies have shown amplification of MDM2 in a proportion of SCCE (14%) (Momand et al., 1998), suggesting that this phenomenon may represent a functional alternative to inactivation of TP53 by mutation. We have also analysed by immunohistochemistry the expression of the cell cycle regulatory proteins p16\(^{INK4a/CDKN2a}\), cyclin E and p27\(^{kip1}\), as well as of cyclooxygenase 2 (COX2). We report that the prevalence and pattern of TP53 mutations in this cohort differ from the ones reported in cohorts from high incidence areas of Europe. In addition, we describe for the first time that P63 is amplified in a significant proportion of SCCE (15.3%).

**MATERIALS AND METHODS**

**Patients and tumours**

Tumour tissues were collected from patients recruited at Hôpital E. Herriot (Lyon, France). All patients were residents in the Lyon area. The criteria for inclusion in the study were (1) presence of a primary SCCE, (2) no primary treatment, (3) signature of an informed consent form. Tissue samples were biopsies collected during endoscopy or samples from surgical pieces. All samples were evaluated by histology. Tumour staging was performed according to the TNM classification (TNM atlas, 4th edition 1997). For biopsies, the stage of the tumour was evaluated by ultrasonography. Clinical charts were reviewed to collect information on the patient’s past medical history, tobacco and alcohol consumption, and follow-up after diagnosis and treatment. The tissue and data collection protocols were approved by local and institutional ethical committees.

**DNA extraction and TP53 mutation detection**

DNA was isolated from microdissected tissue fixed in 10% buffered formalin and embedded in paraffin. After re-hydration, areas of interest were scraped and transferred to extraction buffer (50 µl, Tris-HCl 10 mM pH 9, Protease K 0.1 µg ml\(^{-1}\), Nonidet P40 0.1%) and incubated for 3 days at 56°C with protease K. TP53 exons 5 to 8 were analysed by temporal temperature gradient electrophoresis (TTGE) using the DCODE system (BioRad, Richmond, CA) as described previously (Tanière et al., 2000a). Samples that showed additional and/or abnormal bands were re-amplified from genomic DNA and a second TTGE was performed as a confirmation. Bands corresponding to mutant alleles were cut from the second TTGE, re-amplified using the same primers and analysed by direct sequencing after asymmetric PCR as previously described (Barnas et al., 1997; Tanière et al., 2000a).

**Immunohistochemistry**

Deyparaffinized tissue sections were labelled using standard protocols, after antigen unmasking procedures (3 x 5 minutes in a microwave oven for slides labelled with anti-Cox2, cyclinE and p27\(^{kip1}\) antibodies, and 10 minutes in a pressure cooker for labelling with anti-mdm2). The following primary antibodies were used: CM1 (purified rabbit IgG anti-human p53, 1/500, Novocastra Laboratories Ltd, Newcastle, UK), MDM2 (Ab-1) (monoclonal antibody, clone IF2, 1/100, Calbiochem, San Diego, CA), p16\(^{INK4a/CDKN2a}\) (monoclonal antibody, clone F12, 1/1600, Santa Cruz Biotechnology, Santa Cruz, CA), Cox2 (polyclonal antibody, clone C-20, 1/1000, Santa Cruz Biotechnology, Santa Cruz, CA), cyclin E (monoclonal antibody, clone HE-12, 1/2000, Santa Cruz Biotechnology, Santa Cruz, CA), p27\(^{kip1}\) (monoclonal antibody, clone 1B4, 1/1000, Novocastra Laboratories Ltd, Newcastle). The specificity of the antibodies was checked by Western blot using extracts of oesophageal cancer cell lines TE1, TE6 and TE11 (Barnas et al., 1997; Pluquet et al., unpublished data). Negative immunohistochemistry controls were performed by omission of the primary antibody. Fixed antibodies were detected using either anti-mouse or anti-rabbit biotinylated IgG (1/200 Vectaspin Elite ABC kit, Vector Laboratories Inc.) followed by streptavidin-peroxidase (1/50, 30 minutes at 37°C) and diaminobenzidine-based detection (Vector Laboratories, Inc.). For Cox2 and cyclinE, tumours were considered as positive when at least 10% of tumour cells were labelled. For p27\(^{kip1}\) and p16\(^{INK4a/CDKN2a}\), tumours were considered as negative when less than 10% of the tumour cells were stained.

**Amplification of MDM2 and P63**

Amplifications were detected by differential PCR using the dopamine D2 receptor gene (DRD2) as a reference (Biernat et al., 1997). Genomic DNA was amplified in 25 µl of a reaction mixture containing sense and anti-sense primers for either MDM2 or P63 (20 pmol) and DRD2 (10 pmol), 200 µM of each dNTP, 1 x amplification buffer, 2.5 mM of MgCl\(_2\), and 0.5 µl (2.5 units) of Taq Platinum DNA polymerase (Life Technologies). PCR conditions were: 2 minutes at 94°C, followed by 27 cycles at 95°C for 45 seconds, 55°C (MDM2) or 53°C (P63) for 45 seconds and 72°C for 1 minute with a final extension at 72°C for 5 minutes. The primers used in MDM2 differential PCR were those described in Biernat et al (1997). For P63 differential PCR, we defined the following primers in P63 exon 7 (Genbank accession number AF116762): 5’-CCT ATT TGA ATT ACA TGA TGT GGA T-3’ (sense) and 5’-CAA ACT CGT AAC CCT GTT GTA GA-3’ (anti-sense). A fragment of DRD2 with matched amplification conditions was defined with the following primers: 5’-GGG ATT TTA AGG TTT ACG -3’ (sense) and 5’-GGG ATT TTA AAG TTT ACG GCT AA-3’ (anti-sense). For both differential PCR assays, products were electrophoresed on 3% agarose gels stained with ethidium bromide and analysed by scanning densitometry (BioRad GS-670, Hercules, CA). Each analysis was repeated at least 3 times in independent PCR experiments. A ratio of 2.5 (average of at least three measurements) or above between the specific MDM2 or P63 bands and the DRD2 reference band was regarded as indicative of gene amplification.
RESULTS

Clinical and individual characteristics of the patients

The mean age of the 33 patients included in the study was 61 years (range 45–80 years). 29 were men and 4 were women. 5 patients had developed another tumour several years before the occurrence of SCCE: patient 2 (adenocarcinoma of the colon), patient 4 (malignant T-cell lymphoma), patient 19 (squamous cell carcinoma of the lung) and patients 14 and 23 (squamous cell carcinoma of the head and neck). 2 patients received a liver (patient 1) or renal (patient 9) transplant several years before SCCE. Patient 25 was surgically treated during childhood for congenital mega-oesophagus.

Reliable information on tobacco consumption was available for 23 patients. 22 (96%) were regular smokers (more than 5 pack-years). Information on alcohol intake was available for 21 patients. 17 of them were considered as heavy drinkers (daily alcohol intake over 80 g). All of these 17 patients were heavy smokers.

The pathological staging was available for all resected tumours. For patients for whom only a biopsy was available, the staging was performed by ultrasonoendoscopy. According to the TNM classification, 2 tumours were stage T1, 4 were stage T2, 21 were stage T3 and 3 were stage T4. 19 of the T3 and all T4 tumours showed lymph node involvement (N1). 2 patients (14 and 26) presented 2 distinct infiltrative lesions, and 3 patients (15 and 24) showed multiple dysplastic areas distant from the tumour.

TP53 mutations

A total of 13 TP53 mutations were detected in 12/33 (36%) of the patients. These mutations are listed in Table 1. They are distributed over the exons of the DNA-binding domain (3 in exon 5, 3 in exon 6, 3 in exon 7 and 4 in exon 8). One tumour (12) contained 2 mutations. Of the 11 missense mutations, 3 were transversions and 8 were transitions, 4 of which were C to T occurring within dipyrimidine repeats.

3 mutations gave a null-p53 phenotype (patient 1: nonsense mutation at codon 132; patients 2 and 3: 2-base pair deletion and frameshift at codons 186 and 209, respectively). These 3 tumours were negative for p53 immunostaining. Among tumours with missense mutations, 7/9 were positive for p53 immunostaining.

| Case | Codon | Base change | Amino-acid change |
|------|-------|-------------|-------------------|
| 1    | 132   | AAG→TAG     | Tyr-Stop          |
| 2    | 186   | 2 bp deletion|                   |
| 3    | 209   | 2 bp deletion|                   |
| 4    | 220   | TAT→TGT     |                   |
| 5    | 245   | GGC→AGC     | Gly-Ser           |
| 6    | 245   | GGC→TGC     | Gly-Cys           |
| 7    | 248   | CGG→CAG     | Arg-Gln           |
| 8    | 272   | GTC→ATG     | Val-Met           |
| 9    | 272   | GTC→ATG     | Val-Met           |
| 10   | 273   | CGT→TGT     | Arg-Cys           |
| 11   | 273   | CGT→TGT     | Arg-Cys           |
| 12   | 135   | TGC→TGT     | Cys-Phe           |
| 13   | 218   | GTG→GAG     | Val-Glu           |

Table 1 TP53 mutations in SCCE from a low incidence area of France (Lyon)(a)

Interestingly, tumours 10 and 11 showed opposite staining patterns, although they harboured the same mutation. It is also important to note that 3 tumours without mutations in exons 5–8 were found positive for p53 immunostaining (patients 28, 29 and 31). These tumours may contain a mutation outside the regions analysed.

Amplification of MDM2 and of P63

Amplification of P63 was observed in 5 of the 32 cases tested (15.5%), as detected by P63/DRD2 gene ratios of 2.5 or above (Figure 1). 2 of these tumours also harboured a TP53 mutation.

None of the 27 tumours tested showed MDM2 gene amplification (Table 2). However, many tumours showed mdm2 protein expression detected by immunohistochemistry in a variable proportion of tumour cells. It is important to note that mdm2 is constitutively expressed in cells of the parabasal layers of normal oesophageal epithelium.

Immunohistochemical detection of p16INK4a/CDKN2a, Cox2, cyclin E and p27Kip1

Figure 2 shows typical expression patterns for p16INK4a/CDKN2a, Cox2, cyclin E and p27Kip1. P16INK4a/CDKN2a is constitutively expressed in the basal cell layer of normal oesophageal epithelium. P16INK4a/CDKN2a was detectable in tumour cells in 15/28 (54%) cases tested, but lost in the remaining 13 cases (46%). 5 of these p16INK4a/CDKN2a negative cases also contained a TP53 mutation. Cox2 expression was not detectable in normal oesophageal epithelium. 10/24 (42%) tumours tested were found positive for Cox2 expression (10–80% of cells stained). There was a trend of an association between positivity for Cox2 and advanced tumour stage, since only 1/7 T1, T2 and T3N0 tumours showed Cox2 expression (in 10–20% of the cells), compared with 9/17 T3N1 and T4 tumours (in at least 20% of the cells) (P = 0.069) (based on the 24 samples for which information on stage and Cox2 immunostaining were available, see Table 2). Cyclin E was inconsistently detectable in normal oesophageal epithelium, but was found in at least 10% of tumour cells in 16/23 (70%) cases tested, with no association with the tumour stage. Expression of p27Kip1 was detectable in the parabasal layers of the non-involved epithelium adjacent to the tumour in all the cases. The protein was also...
expressed in 18/24 (75%) tumours tested, with a nuclear localisation, but lost in the remaining cases (25%).

**DISCUSSION**

Our results showed that TP53 mutations in the low-incidence area of Lyon were relatively less frequent (36%) than in high-incidence areas of Europe, where mutation prevalence ranges from 56% (in northern Italy (Esteve et al, 1993) ) to 80% (in Normandy/Brittany (Audrezet et al, 1993; Robert et al, 2000) ). This rather low prevalence of mutations was not compensated by a high prevalence of MDM2 amplification.

Despite its limited size, the present case series also showed a number of other interesting molecular characteristics. In particular, the TP53-related gene P63 was amplified in 5/32 cases (15.5%). This gene has been found to be frequently amplified in squamous cell carcinomas of the lung and of head and neck and is also known as AIS (Amplified In Squamous Carcinomas) (Hibi et al, 1999). The P63 gene encodes a transcription factor that regulates genes that overlap with those controlled by TP53. In contrast with TP53, P63 shows a specific developmental pattern of expression and appears to be required for the normal differentiation of squamous epithelia (Yang et al, 1999). It is important to note that, in this study, we have considered staining in 10% of tumour cells as a threshold for positivity, a criterion more stringent than in several recent studies.

In this cohort of patients, we aimed to identify any association between Cox2 overexpression and advanced tumour stage or poor prognosis. Our results on p16INK4a/CDKN2a and p27Kip1 expression confirm recent data showing that these 2 negative regulators of cell cycle are down-regulated in a variable proportion of SCCE (20–40%) (Itami et al, 1999; Ohashi et al, 1999; Shamma et al, 2000). In
addition, we found that most tumours (70%) expressed elevated levels of Cyclin E. High expression of Cyclin E has been reported in the majority of oesophageal cancer cell lines (Fujii et al, 1998) and in about 30% of primary SCCE (Anayama et al, 1998). Overexpression of Cyclin E has also been observed in preneoplastic lesions and in papillomas in nitrosomethylbenzylamine-induced oesophageal tumorigenesis in rats (Wang et al, 1996).

Overall, these data support the observation that molecules involved in the control of cell cycle progression from G1 to S phase are often altered in SCCE. In gastric cancers, it has been shown that high levels of Cyclin E expression together with low levels of p21 and p27Kip1, correlate with deep invasion. Such a correlation is not seen in our series of cases, as elevated Cyclin E expression was detected in 5/6 T1, T2 and T3N0 tumours, compared to 10/15 T3N1 and T4 tumours. The data available to date do not allow us to evaluate whether there are significant variations in the prevalence of these alterations between different geographic areas.

In many cancers, the pattern of TP53 mutations is informative of the mutagens involved as causal agents. In Figure 3, we have grouped the data reported here with those of the IARC TP53 mutation database. Compared with high-incidence areas, tumours from low-incidence areas show a significantly lower prevalence of transitions and transversions at A:T basepairs, a type of mutation which has been frequently detected in cancers of the oesophagus and of the head and neck, and which may result from the mutagenic action of metabolites of ethanol, such as acetaldehyde. In both high- and low-incidence areas, the main etiological agents implicated so far are the combined consumption of alcohol and tobacco (Launoy et al, 1997, 2000). However, the relative rarity of mutations at A:T basepairs in patients from low-incidence areas, most of whom were heavy drinkers, gives support to the idea that additional factors act as modifiers of the effect of alcohol. Among them, the type of oral microflora and the polymorphism of aldehyde dehydrogenase 2 (ALDH2) have been shown to be important. Indeed, high levels of acetaldehyde in the upper digestive tract are thought to derive from microbial oxidation of ethanol by the oral

Figure 2 Immunohistochemical detection of cyclin E (A), Cox2 (B), p27Kip1 (C) and p16INK4a/CDKN2a (D) in SCCE. (A) More than 50% of the tumour cells were stained in the nucleus with anti-Cyclin E antibody. (B) 20–50% of the tumour cells expressed Cox2 in the cytoplasm. (C) Only 10–20% of the tumour cells expressed the p27Kip1 protein. Lymphocytes and plasma cells were strongly stained and used as a positive control. (D) p16INK4a/CDKN2a expression was lost in tumour cells. In contrast, the non-dysplastic squamous epithelium of the oesophagus showed a focal p16INK4a/CDKN2a expression in the basal cell layers (insert).

Scale: A, B and D (inset): 1 cm = 100 µM; C and D: 1 cm = 500 µM

Figure 3 Comparison between mutation patterns of primary SCCE from high- and low-incidence areas from Europe. Data reported in this article and in the published literature were used (source: IARC TP53 mutation database at http://www.iarc.fr/p53; version R5, June 2001) were used. High-incidence areas: Normandy/Brittany and northern Italy (77 mutations); Low-incidence areas: Lyon (France), Paris (France) and Lausanne (Switzerland) (39 mutations)
microflora. The composition and quantities of the oral microflora may vary from one area to another and therefore influence the actual levels of acetaldehyde that can damage the oesophageal mucosa (Muto et al., 2000b). Furthermore, it is important to note that a polymorphism in ALDH2 has been found to be associated with a higher risk of head and neck and of oesophageal cancers (Muto et al., 2000a). Whether these factors contribute to explain the variations in incidence of SCC in Western Europe awaits further evaluation. It will also be important to evaluate whether the pattern of TP53 mutations in other cancers related to alcohol intoxication, such as oral cancer and hepatocellular carcinomas, also vary from one geographic area to another.

ACKNOWLEDGEMENTS

We thank JC Boulez, C Partensky and E Tissot (Hôpital Edouard Herriot) for providing surgical specimens; JA Chavyaille (Hôpital Edouard Herriot) for providing biopsies; R Montesano and J Hall for critical comments and helpful suggestions, M Olivier for technical assistance with immunostaining and M Wrisez for secretarial assistance.

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