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ONCOLOGY

Multiple head and neck tumours and their genetic relationship

Relazioni genetiche dei tumori multipli della testa e del collo

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SUMMARY

Second primary tumours represent one of the major causes of failure in the treatment of head and neck cancer. Advances in early diagnosis and treatment have improved the patient's disease-specific survival. However, the increase in the occurrence of second primary tumours negatively influences the patient's chance of long-term survival. To understand the molecular events underlying the appearance of head and neck multiple tumours, the clinical history has been evaluated in 2 patients both of whom developed 3 primary tumours of the head and neck. To establish the genetic relationship between the different head and neck cancers which had developed in these 2 patients, loss of heterozygosity was investigated using microsatellite markers located on chromosomes 3p, 9p, 11q, 13q, and 17p. These markers were selected as they frequently demonstrate loss of heterozygosity in head and neck cancer. The following markers were used: D3S1234, D3S1300, D9S170, D11S490, and D17S158. Primer sequences were obtained from the genome database for all of these markers. The third tumour that developed in the first patient, 13 years after the primary, showed loss of heterozygosity on chromosome 17p (in the locus for the gene TP53), which was not present in the previous tumours. All tumours in the second patient showed heterozygosity of chromosome 11 at the locus D11S490. These 2 cases show that multiple tumours can be derived from a genetic alteration of a subclone from previous tumours or from an independent preneoplastic cell clone present in the head and neck mucosa.

KEY WORDS: Head and neck • Malignant tumours • Second primary tumours • Multiple tumours

RIASSUNTO

La comparsa di secondi tumori primitivi delle prime vie aeree rappresenta una delle cause di insuccesso nel trattamento dei tumori della testa e del collo. I soddisfacenti risultati raggiunti nel controllo della malattia primitiva e la diagnosi precoce hanno migliorato i tempi di sopravvivenza e per tale ragione si assiste ad un aumento di incidenza di secondi tumori primitivi. Al fine di comprendere i meccanismi molecolari che intervengono nella comparsa di secondi tumori dello stesso distretto abbiamo preso in considerazione la storia clinica di due pazienti che hanno sviluppato ciascuno 3 tumori primitivi della testa e del collo. Al fine di verificare in questi pazienti la relazione clonale tra i tumori delle diverse sedi è stata condotta una analisi microsatellitare di markers che identificano loci cromosomici frequentemente interessati da LOH nei carcinomi della testa e del collo che riguardano i cromosomi 3p, 9p, 11q, 13q e 17p. Dai risultati è emerso che il terzo tumore sviluppato dal primo paziente a distanza di 13 anni dalla comparsa del primo tumore, mostrava la perdita di eterozigosità sul cromosoma 17 in corrispondenza del gene TP53. Nel caso del secondo paziente, tutti i tumori hanno dimostrato una eterozigosità per il locus D11S490. Dai risultati ottenuti nei due casi da noi studiati i tumori multipli della testa e del collo si sono sviluppati sia per ulteriore mutazione di un unico clone comune sia come espressione di foci multipli a potenzialità neoplastica presenti nella mucosa delle prime vie aeree e quindi da un clone cellulare di origine indipendente.

PAROLE CHIAVE: *Testa e collo • Tumori maligni • Secondi tumori primitivi • Tumori multipli*

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Introduction

Second primary tumours (SPTs) represent one of the major causes of failure in the treatment of head and neck cancer. Advances in early diagnosis and treatment have improved the patient's disease-specific survival. However, the increase in the occurrence of SPTs at a rate of 2-3 new cases per year [1,2] adversely affects the patient's chance of long-term survival. Patients at higher risk of developing an SPT are those affected by early cancer with a long-term survival probability.

According to the definition of Slaughter and Southwick [3], who introduced the term "field cancerization", SPTs develop independently from the primary tumour because of the widespread exposure to carcinogens in the upper aerodigestive tract mucosa. Slaughter and Southwick found that all of the epithelium beyond the boundaries of the

tumour showed histologic changes, suggesting that the mucosa of the head and neck undergoes a change, perhaps because of carcinogen exposure, and is, therefore, more susceptible to the development of many foci of malignant transformation. When proposed, there was no molecular basis for this hypothesis, but the term field cancerization has been used since then to describe 3 events: 1) a wide field of upper airways mucosa that tends to show pre-malignant disease, 2) the frequency of multiple primary tumours in epithelial areas affected by widespread pre-malignant lesions, and 3) the possibility of developing distant related primary tumours in the upper aerodigestive tract.

It is now known that head and neck cancers develop through a multistep process of genetic alterations to oncogenes and tumour-suppressor genes. The development of new molecular biology techniques has made it possible to identify chromosomal regions affected by tumour-suppressor genes frequently involved in the tumorigenesis of head and neck cancers [4-8]. Recent molecular genetic studies have raised doubts about the theory of Slaughter and Southwick by showing that mucosal fields with morphologically normal cellular clones but genetic alterations can be as wide as 7 cm in diameter [9 10]. Some models have been proposed to support a common clonal origin of an SPT based on the hypothesis that pre-neoplastic cells can be replaced in other sites through the saliva [9-12] or by later migration [13-15].

It is not clear whether SPTs derive from the same cellular clone from which the primary tumour develops or whether they develop independently from genetically modified cellular clones present in the mucosa because they are exposed similarly to carcinogens. This uncertainty has led to a review of the definition of SPTs [16] based on clinical criteria that consider the distance and time between the appearance of the primary and secondary tumours. Braakhuis et al. [17 18] proposed a new classification that introduces the term "second fields tumours" (SFTs) to define a second tumour with the same genetic profile as the primary tumour without considering the time and the distance of appearance. In contrast, SPT refers to a second primary tumour with different genetic and clinical characteristics from those of the primary tumour.

To understand the molecular events causing the onset of multiple head and neck cancers and their clonal relationship, the present investigation focused on 2 patients, each of whom developed 3 head and neck cancers. This is a rare event that has not been described before. To better understand the genetic relationship between the cancers, a microsatellite analysis was performed of the patients' first, second, and third tumours by analysing changes in the chromosomal loci on chromosomes 3p, 9p, 11q, 13q, and 17p, which occur frequently due to loss of heterozygosity (LOH) in head and neck tumours [19]. Particular attention was focused on the chromosomal locus 17p, which is located on the TP53 gene and codifies a nuclear *phosphor-protein* that acts as a tumour suppressor. An alteration in the TP53 gene is present in most head and neck pre-malignant and malignant lesions, suggesting that this change plays an important role in the early steps of head and neck carcinogenesis [20].

Materials and Methods

Patient P.P. a 39-year-old male presented, in 1993, with a squamous cell carcinoma (SCC) of the larynx (stage T3N0M0). He was submitted to total laryngectomy, and the histological examination of the surgical specimen revealed SCC, moderately differentiated, of the true vocal folds with extension to the anterior commissure, ventricle, and left vocal fold; the resection margins were free of disease. In 2002, 9 years after the primary diagnosis, he developed a SCC of the soft palate (T2N0M0) and underwent uvulopalatoplasty. Histological examination of the surgical specimen showed a moderately differentiated SCC with resection margins free of disease. Because of the high risk of recurrence of tumours in the oral cavity and oropharynx [7 15 21] and because this was a SPT, it was decided to perform adjuvant radiotherapy with the lateral parallel-opposed photon field encompassing the surgical bed (total dose 60 Gy/6 weeks). Finally, in 2006, during a regular follow-up, P.P. presented with a SCC of the hypopharynx (T3N0M0), which was treated with 2 cycles of chemotherapy of 100 mg/m^2 of cisplatinum (for 1 day) and 1000 mg/m^2 of 5-fluorouracil (for 5 days) during weeks 1 and 4. At the end of the second cycle of chemotherapy, the mass had significantly decreased in size and it was decided to operate. The patient died of disease progression in 2007.

Patient C.A., a 70-year-old male presented, in 1996, with SCC of the left true vocal fold (T1N0M0) and he underwent cordectomy. Histological examination of the surgical specimen showed SCC and moderate dysplasia with free surgical margins. In 1998, the patient developed SCC in the right margin of the mobile tongue (T1N0M0), which was treated with surgery alone. In 2004, he developed a lesion of the right retromolar trigone, and histological examination of the biopsy specimen revealed a SCC (T1N0M0), which was treated with radiotherapy (total dose 74 Gy/7 weeks). The patient is still alive and in good health and is free from the disease.

DNA samples

Patients were informed about the study and gave their informed consent to use their paraffin-embedded tumour specimens. Blood samples obtained from the 2 patients served as the control material.

DNA was extracted from the surgical material or biopsies of the laryngeal cancer, soft palate cancer, and hypopharyngeal cancer taken from patient P.P. and from the true

vocal fold cancer, right tongue margin cancer, and retromolar trigone cancer taken from patient C.A. The DNA extracted from the tissue specimens of the excised lesions was purified from 3 x 10 µm paraffin-embedded sections using commercial kits (Wizard Promega, Madison, WI, USA and GEH, Amersham, Buckinghamshire, UK). Normal DNA was purified from lymphocytes isolated from the patients' peripheral blood samples using a Nucleon HT kit (GEH, Amersham, Buckinghamshire, UK).

Microsatellite analysis

The LOH was assigned using 5 microsatellite markers located on chromosomes 3p, 9p, 11q, 13q, and 17p. The following markers were used: D3S1234 (FHIT), D3S1300 (FHIT), D9S170 (PAPPA), D11S490 (C1pB), and D17S158 (TP53).

Primer sequences were obtained from the Genome Database (http://gdbwww.gdb.org/).

One primer (MWG) of each marker was end-labelled with FAM fluorescent dye.

PCR was performed in a standard reaction mix using AmpliTaq Gold (PerkinElmer, Inc., Waltham, MA, USA) PCR products were run on an ABI-3100 Automated Fluorescent Sequencer Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) with a GeneScan™ 500 ROX size standard (Applied Biosystems, Carlsbad, CA, USA), and the data were analysed using the manufacturer's GeneScan 2.1 software.

LOH was scored if 1 allele demonstrated a reduction greater than 50% in the tumour sample when compared with the same allele in the control DNA.

Results

Patient P.P.

LOH for the markers used in the study was not detected in the primary tumour (laryngeal cancer) or second tumour (soft palate cancer) (Fig. 1). However, LOH was detected for the locus of the TP53 gene in the third tumour (hypopharyngeal cancer) (Fig. 2).

Patient C.A.

LOH for the locus of the C1pB gene was detected in all 3 tumours: the primary tumour (true vocal fold cancer), second tumour (tongue cancer), and third tumour (retromolar trigone cancer) (Fig. 3).

Discussion and Conclusions

In patient P.P., the occurrence of the second tumour (soft palate cancer) appeared 9 years after the primary (laryngeal cancer) and was located > 2 cm from the primary tumour. These characteristics correspond to the clinical definition of an SPT but the tumours were genetically related and they could be defined as SFTs. The third tumour

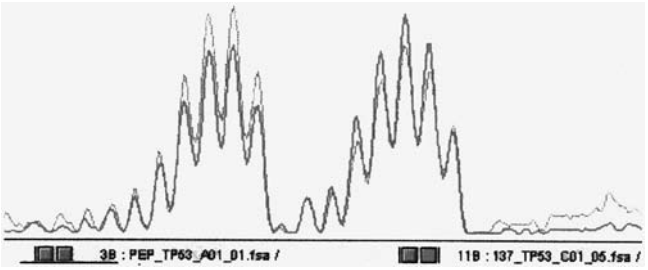

Fig. 1. Patient P.P. informer marker D17S158 ■ DNA from blood ■ DNA from primary tumour (laryngeal cancer).

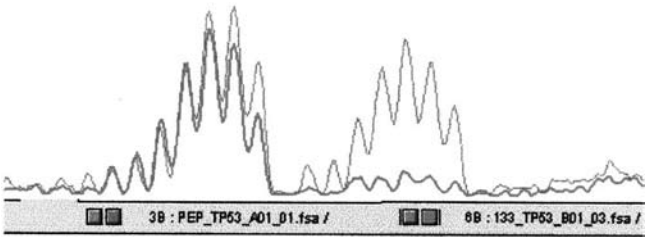

Fig. 2. Patient P.P. informer marker D17S158 ■ DNA from blood ■ DNA from third tumour (hypo-pharyngeal cancer).

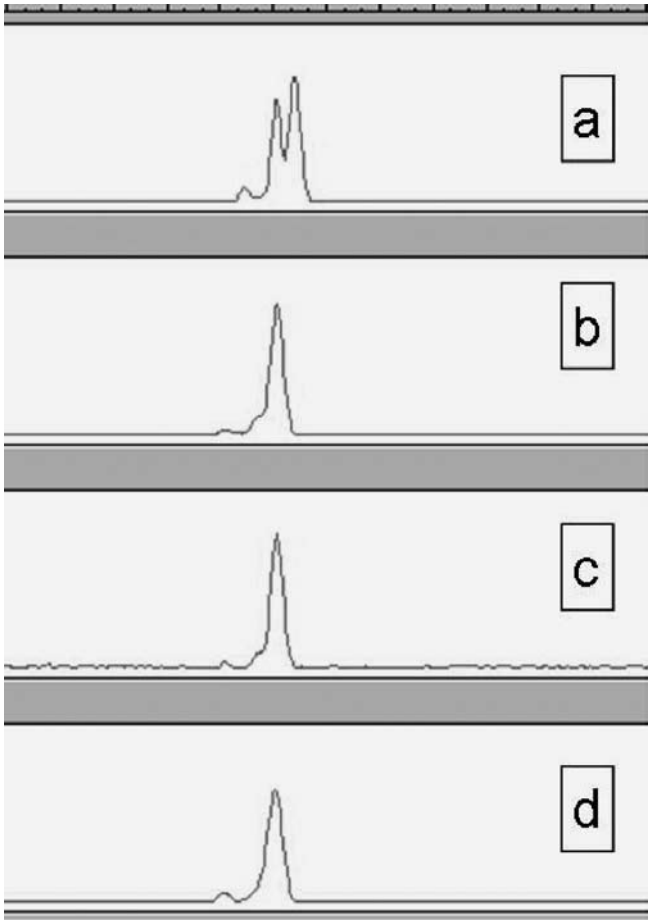

Fig. 3. Patient C.A. informer marker D11S490: a) DNA from blood, b) DNA from vocal fold cancer, c) DNA from tongue cancer, d) DNA from retromolar trigone cancer.

(hypopharyngeal cancer) occurred 13 years after the primary tumour and 4 years after the second tumour, and showed an LOH for the locus of the TP53 gene. The TP53 gene mutation seems to be an early event during the multistep process of head and neck cancer. This appears to confirm the hypothesis that this last tumour had an independent origin from the other two and can be defined as an SPT, although we cannot exclude the possibility that this discordance represents a divergence that occurred during genetic evolution of the tumour and that the tumours originated from a common clone [11].

In patient C.A., the second tumour (mobile tongue cancer) occurred a little earlier than 2 years after, and at a distance of > 2 cm from, the primary tumour (vocal fold cancer), and the third tumour (retromolar trigone cancer) appeared 6 years after, and at distance of < 2 cm from, the second tumour. Microsatellite analysis showed a common genetic alteration comprising an LOH for the locus of the C1pB gene. The presence of a common genetic pattern, in this patient, led us to define the second and third tumours as SFTs and to conclude that the 3 tumours had a common origin.

To explain the common clonal origin of the multiple tumours, various modalities have been proposed, such as shedding of genetically mutated cells into the saliva and implantation at other sites or lateral migration. The development of these malignant clones might take an extended time, perhaps several years or more [7 9 11].

No others cases of patients with 3 metachronous multiple tumours located in the head and neck have been reported. A study of 10 patients by Tabor et al. [21] found 2 patients each with 3 synchronous and metachronous multiple head and neck cancers. They found little evidence of a single genetic precursor for these multiple tumours although they found a common clonal origin in 6 of the 10 patients. Bedi et al. [11] studied 8 patients with multiple head and neck tumours and observed 2 cases each with 3 synchronous localizations but failed to demostrate a genetic relationship between the tumours and concluded that some SPTs may arise as independent lesions that probably share a common origin.

The origin and the mechanism of occurrence of head and neck multiple tumours remain controversial issues. Some Authors believe that these tumours are derived from a common cell clone that develops a second tumour at a distance by migration, whereas others believe that they have an independent origin because of the large and diffuse pre-cancerous lesions present in the mucosa of the upper airways (i.e., field cancerization). In our opinion, both mechanisms could occur in the same patient possibly because of an individual's predisposition to genetic mutations after widespread epithelial exposure to carcinogens, which may result from a deficiency in the DNA repair mechanisms [22]. This could explain the occurrence of multiple tumours caused both by the migration of cancerous cell clones and independently by different mutated cell clones.

The recent trend to early diagnosis and long-term follow-up has revealed that patients affected by head and neck cancer are predisposed to developing multiple tumours. Further studies are needed to better understand the mechanisms responsible for the occurrence of multiple head and neck cancer and to obtain new strategies for treatment.

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