Aurora-A overexpression as an early marker of reflux-related columnar mucosa and Barrett’s oesophagus

V. Agnese1†, D. Cabibi2†, D. Calcara1, M. Terrasi1, G. Pantuso3, E. Fiorentino3, C. Intrivici1, G. Colucci4, F. Aragona2, N. Gebbia1, V. Bazan1 & A. Russo1*

1Section of Medical Oncology, Department of Surgery and Oncology; 2Department of Histopathology; 3Section of Surgical Oncology, Department of Surgery and Oncology, Università di Palermo, Palermo; 4Division of Medical Oncology, National Institute of Oncology, Bari, Italy

Background: The development of oesophageal adenocarcinoma is generally closely associated with the presence of a specialised intestinal-type epithelium such as that found in Barrett’s oesophagus (BO). A particular histological condition is when the distal oesophagus showing cardiac and/or fundic mucosa without intestinal metaplasia cannot be defined as ‘Barrett’s mucosa’ (condition that we call ‘columnar-lined oesophagus’ (CLO)) and up till now, there has been no agreement in literature about the management of this condition. Aurora-A overexpression leads to centrosome amplification, chromosomal instability and aneuploidy in mammalian cells.

Patients and methods: A prospective study was carried out on 28 consecutive patients who presented columnar mucosa above the gastro-oesophageal junction (GOJ) at endoscopy. As controls, two more biopsies were obtained, one on the normal-appearing squamous oesophagus above the GOJ, as far as possible from the columnar mucosa (controls A), and one taken 1 cm below the GOJ (controls B). The Aurora-A and p53 expression levels were analysed respectively by Quantitative Real Time PCR and immunohistochemistry.

Results: Twelve patients were affected by BO (43%) while the other 16 patients (57%) had a CLO. Nine of 28 (32%) cases were focally positive for p53 immunostaining. All the BO/CLO samples were positive for the Aurora-A transcript with regard to controls. Furthermore, 13 of 28 (46%) cases showed overexpression (above the median for the whole group).

Conclusion: Due to the low number of cases, we are not at present able to state that statistically significant quantitative differences in Aurora-A messenger RNA expression exist between CLO and BO cases with and without dysplasia and p53-positive immunostaining. Further studies on a larger number of cases with a follow-up period are necessary in order to establish the risk of progression and the correct management of these subjects.

Key words: Aurora-A overexpression, Barrett’s oesophagus, cell cycle, columnar-lined oesophagus, p53 protein

Introduction

In recent years, there has been an estimation in North America and Europe of a 40-fold increase in the risk of developing invasive adenocarcinoma of the oesophagus¹. The proposed model of histological progression of this kind of tumour is a stepwise progression recognised as a metaplasia-dysplasia-adenocarcinoma sequence (MCS) [2]. Moreover, the development of oesophageal adenocarcinoma (OA) is generally closely associated with the presence of a specialised intestinal-type epithelium such as that found in Barrett’s oesophagus (BO) [3, 4]. BO occurs in 10%–12% of patients with chronic gastro-oesophageal reflux. Over a period of time, the presence of this stressful agent results in replacement of the normal squamous epithelium by the more acid-resistant columnar epithelium [5]. For this reason, Barrett’s mucosa is defined as the endoscopic presence of a metaplastic mucosa with ‘goblet cells’ in the oesophagus, regardless of the length of the segment [6]. A particular histological condition is when the distal oesophagus showing cardiac and/or fundic mucosa without intestinal metaplasia (IM) cannot be defined as Barrett’s mucosa [7] and up till now, there has been no agreement in literature about the management of this condition. Hereafter, we refer to this condition as columnar-lined oesophagus (CLO).

As in the molecular model proposed for colorectal cancer progression [8], several genetic alterations have been reported in the MCS [9]. In particular, p53 alterations have been reported in 5%–10% of cases with indeterminate dysplasia, in 65% of those with low-grade dysplasia, in 75% of cases with...
A prospective study was carried out on 28 consecutive patients, Helicobacter pylori (Hp) free and with no history of previous Hp infection, who presented columnar mucosa above the gastro-oesophageal junction (GOJ) at endoscopy. The GOJ was identified as the point at which the tubular oesophagus changes to become a sack-like structure. The presence of hiatus hernia was noted. The patients had been referred to the Oesophageal Surgical Unit, Department of Oncology, University of Palermo for an evaluation of their suspected gastro-oesophageal reflux disease (GORD). They all complained of one or more symptoms such as heartburn, regurgitation, dysphagia, otolaryngological symptoms and asthma.

### Tissue handling

After informed consent, four-quadrant biopsies every 2 cm, beginning at the top of the endoscopic segment of the columnar mucosa above the GOJ, were taken from each patient and processed within 30 min of biopsy. As controls, two more biopsies were obtained, one on the normal-appearing squamous oesophagus above the GOJ, as far as possible from the columnar mucosa (Controls A), and one taken 1 cm below the GOJ (Controls B). All the histological analysis was carried out by one of the authors (CD). All biopsies were bisected, one half of each sample was processed for pathological examination, and the remaining half of the sample pool was immediately frozen and stored at −80°C for subsequent biomolecular analysis. For the pathological examination, the samples were fixed in 10% buffered formalin and embedded in paraffin and 5 μ sections were obtained at five different levels and stained with haematoxylin and eosin and Alcian blue periodic acid–Schiff (PAS) stain, in order to facilitate the identification of goblet cells. IM was defined as the presence of intestinal-type goblet cells stained blue with Alcian blue PAS stain.

### p53 immunostaining

Immunohistochemical studies were carried out by means of a avidin–biotin complex technique. The primary monoclonal antibodies used in this study were p53 (clone DO7) obtained from Novocastro laboratories. Sections were cut from 10% formalin-fixed, paraffin-embedded materials and then deparaffinised in xylene and re-hydrated through alcohol. The immunohistochemical assay was carried out according to the manufacturer’s instructions of Universal LSAAB (Dako, Glostrup, Denmark). In order to improve the immunostaining, samples were digested with 0.1% trypsin and microwaved in 10 mM citrate buffer (pH 6.0) before incubation. As a positive control for p53, the immunostaining was carried out on sections of rectal adenocarcinoma which had proved to be positive in previous assessments for the above-mentioned antibodies. Finally, negative controls without primary antibodies were included in each run of immunohistochemistry. We considered a nuclear staining pattern as positive for p53 immunostaining.

All cases were revised by the two pathologists (CD and AF) and only cases in which there was interobserver agreement about the presence of low-grade dysplasia were considered as dysplastic. Moreover, in keeping with previous reports on p53 values in interobserver agreement of low-grade Barrett’s dysplasia diagnosis and in the correlation with disease progression, the presence of at least focal p53 expression was used to confirm the low-grade dysplasia [30–32].

### RNA extraction and complementary DNA synthesis

Total RNA was extracted with the use of the Rneasy MiniKit (Qiagen, Hilden Germany) according to the manufacturer’s instructions. The extracted RNA was stored at −80°C until further use. RNA integrity was verified on the Agilent 2100 bioanalyzer with the use of the RNA 6000 Nano assay protocol (Agilent Technologies, Palo Alto, CA). Only samples with an RNA integrity number between 8 and 10 were analysed. For complementary DNA (cDNA) synthesis 5 μg of total RNA were reverse transcribed in a final volume of 50 μl with a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The samples were incubated for 10 min at 25°C and for 2 h at 37°C on the GeneAmp 9700 Applied Biosystem (Applied Biosystems).

### Materials and methods

#### Patient features

A prospective study was carried out on 28 consecutive patients, *Helicobacter pylori* (Hp) free and with no history of previous Hp infection, who presented columnar mucosa above the gastro-oesophageal junction (GOJ) at endoscopy. The GOJ was identified as the point at which the tubular oesophagus changes to become a sack-like structure. The presence of hiatus hernia was noted. The patients had been referred to the Oesophageal...
quantitative determination of Aurora-A by Real Time RT-PCR

Real Time RT-PCR was carried out with the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using the TaqMan method. Quantification was carried out with the use of the threshold cycle (Ct) value. For the detection of Aurora-A and for normalisation the following predesigned primer and probe set were used: assay-on-demand Gene Expression Product, number Hu01590514_m1, AURKA and assay-on-demand Gene Expression Product, number 4333763F, Hu-PPIA, (Applied Biosystems). For the PCR, 100 ng of cDNA in a final volume of 50 µl of TaqMan Universal PCR Master Mix (Applied Biosystems) was used according to the manufacturer’s guidelines. Each sample was analysed in triplicate and the mean quantity of each triplicate calculated by the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). In our study, the comparative Ct method was used to quantify the relative gene expression with the formula $2^{-\Delta\Delta Ct}$, using cyclophilin A (Hu-PPIA) as the endogenous control. Two different control samples were used as calibrators. Specifically, for each patient the analysis was conducted in triplicate by comparing the Aurora-A expression levels of columnar mucosa above the GOJ, respectively toward normal-appearing squamous oesophagus (control A), with those 1 cm below the GOJ columnar mucosa (control B) and then making a comparison between the two controls.

results

histological and p53 immunohistochemical results

Table 1 shows all the pathological characteristic of the biopsies investigated. Patients were, principally, male (19/28, 68%) with a mean age of 46 (range 27–61). Twelve patients were affected by BO (43%) (Figure 1A) while the others 16 patients (57%) had a CLO. All the CLO samples lacked Alcian blue-positive goblet cells at histology (Figure 1B) and weak Alcian blue positivity was present in a few columnar cells only in some cases (Figure 1C). The control A cases consisted of histologically normal-appearing squamous mucosa. The control B samples consisted of normal-appearing fundic mucosa without goblet cells (Figure 1D). Nine of 28 (32%) cases [five of 12 BO (42%) and four of 16 CLO (25%)] showed some histological alterations such as pale and inconspicuous cytoplasm of the columnar cells; reduction of the cytoplasmic mucous production and enlarged, rounded, vesicular nuclei with evident nucleoli, occurring without inflammation and indicative of the so called ‘low-grade dysplasia type II’ or ‘hyperplastic dysplasia’. They were focally positive for p53 immunostaining (Figure 1E, F and G). Control A cases showed mild p53-positive immunostaining only in the basal layer of squamous epithelium. Control B cases were devoid of dysplastic alterations and were p53 negative (Figure 1H).

Aurora-A mRNA expression

RNA extracted from 28 samples of columnar mucosa ‘1 cm above GOJ’ (consisting both of BO and CLO samples) and from the two controls was analysed for Aurora-A messenger RNA expression by Real Time RT-PCR. All the BO/CLO samples were positive for the Aurora-A transcript with regard to controls. In particular, the comparison of the two controls showed the same Aurora-A expression value close to 1 for all samples while all the BO/CLO samples had a higher value.
Aurora-A expression level when compared both to the control A and to the control B cases. Furthermore, 13/28 (46%) cases showed overexpression (above the median for the whole group). Figure 2 shows an example of Real Time RT-PCR experiment of Aurora-A expression respectively in 1 cm above GOJ columnar mucosa case and control A, belonging to the same patient and the peptidylprolyl isomerase A (PPIA) expression levels of all samples included in this study.

**discussion**

Most oesophageal tumours are adenocarcinomas (OA) which generally arise from a MCS. Although this sequence has been fully studied and characterised, a small percentage of OA arise from BO. This is a precancerous form associated with chronic GORD where squamous epithelium is replaced by metaplastic columnar epithelium. The natural history of the sequence BO-OA is not well known but it is likely that the store of multiple genetic alterations gives rise to Barrett’s oesophagus-a non-invasive neoplasia-oesophagus adenocarcinoma (BO-NIN-OA) sequence. The diagnosis of BO is mainly based on the presence of IM and goblet cells [33]. Nowadays, although it is well known that BO-carrier patients show 30–125 times more risk for developing OA than the normal population, it is still difficult to discriminate the BO patients with a major risk of developing OA. The application of a rigid follow-up of BO patients after the first endoscopic–histological diagnosis is the only way to identify the presence of OA at an early stage. In certain patients, moreover, for instance in those with CLO, the absence of intestinal goblet cells does not permit the inclusion of such patients in follow-up programmes, which gives rise to an unclear clinical management of these cases. For all these reasons, the aim of this study is to identify the presence of Aurora-A and p53 alterations in specific forms such as CLO and BO with the object of assessing in these forms the biomolecular alterations that precede the histological phenotype and that may help in the choice of the clinical management of such cases. These analyses have been conducted not by means of the identification of the genetic alteration but by the study of the different Aurora-A mRNA and p53 protein expression levels, in order to determine their effect directly on the RNA and/or protein.

Several sporadic tumours often show chromosomal aberrations that precede genetic alterations and that might give rise to histological changes and then to cancer progression. The presence of these chromosomal alterations has, in fact, been related to the invasive tumour. The chromosomal aberration occurs principally in dividing cells during the mitotic event when the chromosomes are aligned in a metaphasic plate and are about to be segregated into two daughter cells. Each step of this multiphase process is strictly regulated by several checkpoints and by different proteins with a specific role in chromosome condensation, alignment and segregation. The alterations in mitosis will give rise to abnormal chromosome segregation and then to cells with

![Amplification Plot](Aurora-A and p53 expression levels in Barrett’s oesophageal (11BO) and control A (11CA) of the case number 11; B) Cyclophillin A (peptidylprolyl isomerase A [PPIA]) expression levels in all 22 samples analysed.)
aberrant genetic panels. This study focused on the analysis of two proteins playing two important key roles in the cell cycle: Aurora-A, also known as ‘a guardian of the pole’, and p53, previously known as ‘guardian of the genome’. The first is a serine threonine kinase which, when activated by phosphorylation, is involved in several phases of mitosis such as centrosome maturation, centrosome separation, mitotic entry, bipolar spindle assembly and chromosome alignment on the metaphase plate [34]. The Aurora-A gene is located at chromosome 20q13, which is commonly amplified in various epithelial malignant tumours, including breast, colon, bladder, ovarian and pancreatic cancer and the levels of Aurora-A mRNA and protein are also increased in such tumours [34].

Contrasting data are reported on the Aurora-A role in the histological grade of human tumours [35–37]. Some studies, in fact, have indicated that Aurora-A overexpression is significantly associated with higher grade tumours and poor prognosis, while others report that the activation and overexpression of Aurora-A is more frequently detected in early-stage human ovarian cancers. Up-regulation of Aurora-A mRNA and protein were analysed by semiquantitative reverse transcription PCR and immunohistochemistry in patients affected by human oesophageal squamous cell carcinoma (OSCC) and in paired normal tissues [27, 38]. These studies have reported that Aurora-A expression, in terms of both mRNA and protein, is elevated in OSCC tissues and that it is correlated with distant lymph node metastasis and tumour invasion. Furthermore, Tanaka et al. [20] report that the up-regulation of Aurora-A protein is an independent prognostic factor. Our study is the first study in which the different Aurora-A expression level is analysed in the precancerous form of the oesophagus such as in BO and in CLO, a condition probably preceding BO.

The presence of IM with goblet cells is considered the most significant change for the diagnosis of BO, a fact emphasised by the dictum ‘no goblets, no Barrett’s’ [33]. The meaning of CLO devoid of goblet cells or showing only a few Alican blue positive, columnar cells in the malignant transformation of BO is still debated [39–42] and there will probably be no follow-up of these patients. Sometimes, CLO has been considered a function. Oncogene 2002; 21(40): 6175–6183.


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