CYP2A6/2A7 and CYP2E1 expression in human oesophageal mucosa: regional and inter-individual variation in expression and relevance to nitrosamine metabolism

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Oesophageal cancer is one of the most common and lethal malignancies in the world. Despite many efforts, treatment is still ineffective for most cases; thus, the development of preventive strategies is crucial for decreasing the burden presented by this disease. Environmental factors, particularly nitrosamines, are thought to be involved in the genesis of oesophageal tumours, and knowledge about the expression of enzymes capable of activating pre-carcinogens in human oesophagus is very important for the development of preventive measures. We analysed the expression of CYP1A1, CYP1A2, CYP2A6/2A7, CYP2E1 and CYP3A4 mRNA in oesophageal mucosa of 50 patients by semi-quantitative RT-PCR. In five patients, who suffered from squamous cell carcinoma, we measured N-nitrosodimethylamine and N-nitrosothiethylamine metabolism in normal and tumorous tissue. CYP2A6/2A7 mRNA was expressed in 61% and CYP2E1 mRNA in 96% of the patients, but in the latter a lower degree of inter-individual variation was observed. These enzymes were expressed either in the distal or middle portions of the oesophagus of 90% of the patients. CYP1A1, CYP1A2 and CYP3A4 mRNA expression was not detected in any portion of the oesophagus. Oesophageal microsomes activated N-nitrosodimethylamine with a low degree of inter-individual variation and microsomes prepared from the tumour of a patient who strongly expressed CYP2A6/2A7 mRNA activated N-nitrosodiethylamine. We conclude that the human oesophagus expresses CYP2A6/2A7 and CYP2E1 and can activate nitrosamines. Notably, the expression of these enzymes is preferentially localized to the most common sites where tumours arise.

Introduction

Cancer of the oesophagus is the ninth most frequent and the sixth most fatal malignancy in the world (1,2). It occurs predominantly as squamous cell carcinoma and adenocarcinoma, with the vast majority of tumours arising in the distal and middle portions of the oesophagus (3,4). Most cases are detected only at a later stage and, despite efforts to improve treatment, the prognosis is still very poor, making this malignancy highly lethal (4,5). The burden also comes from the increase of oesophageal cancer incidence rates in some regions of the world. In the USA, for instance, where oesophageal cancer ranks seventh in mortality among men, and ninth among women, adenocarcinoma incidence rates have had a steady increase by up to 10% a year in the last years (6).

One particular characteristic of this malignancy is the geographical variation in incidence around the world, which suggests the influence of environmental factors (7). Studies analysing mutations in the TP53 gene support the hypothesis that oesophageal cancer is highly associated to environmental carcinogens (8). In the West, where the observed incidence rates are not as high as in the East, the main risk factors associated with oesophageal cancer are heavy alcohol consumption and tobacco smoking (7). Although tobacco smoke contains many different carcinogens, nitrosamines are the only ones that can easily induce oesophageal tumours in different species of experimental animals. The role of alcohol is still not definitely understood (9), but ethanol has been shown to increase nitrosamine activation into ultimate carcinogens (10). Alternatively, many authors have shown that ethanol can change the organotropism of nitrosamines from liver to the oesophagus (11–16).

In the East, on the other hand, environmental and nutritional factors have been associated with the disease and nitrosamines have been suggested to be involved in the initiation of the tumours (17,18). Furthermore, O6-methylguanine, an adduct formed from nitrosamines, has been found in DNA of oesophageal tumours of patients who live in high incidence areas of the disease (19).

Nitrosamines are precarcinogens and therefore require metabolic activation, catalysed by cytochrome P450 (CYP) enzymes, to be able to carry out their carcinogenic effects. In the rat, the oesophagus is the main target for tumour induction by nitrosamines (20). This susceptibility is considered to be, in part, due to the presence of locally expressed CYP enzymes capable of efficiently catalysing the activation of several nitrosamines, such as N-nitrosodiethylaniline (NDEA) (12,21–23), N-nitrosomethylbenzylamine (NMBzA) (24), N-nitrosomethylamylamine (NMAA) (22,25), and N-nitrosomethyl-
We have recently shown that rat oesophageal and hepatic microsomes activated NDEA with different kinetic constants, and that the oesophageal, but not the hepatic metabolism of NDEA was catalysed in great part by CYP2A3 (23). Nevertheless, information about the expression of CYP enzymes capable of activating nitrosamines and other pre-carcinogens in human oesophagus is limited. CYP1A1, CYP2E1 and CYP3A have been shown to be expressed in human oesophagus at the mRNA and protein levels (27–30), and recently CYP2A protein has also been shown to be present in human oesophagus of some individuals (27). As oesophageal cancer is practically incurable, it would be very important to develop preventive strategies by knowing which carcinogens can be activated in individuals from different populations, and if, in similarity to the rat, the human oesophagus is susceptible to nitrosamines.

Since the oesophagus is constantly exposed to carcinogens derived from the diet, we have analysed the expression of the main CYP enzymes capable of metabolizing nitrosamines and other pre-carcinogens in the oesophageal mucosa of patients from Brazil, one of the high incidence areas for the disease in the Western world (2). In this work, we show that among the main carcinogen metabolizing CYP enzymes, only CYP2A6/2A7 and CYP2E1 are expressed in the oesophageal mucosa. Strikingly, the expression is preferentially localized either to the distal or medium portions of the oesophagus, the main sites where cancer arises.

**Materials and methods**

**Chemicals**

Trizol® reagent and random primers were purchased from Gibco BRL, USA. [4-14C]Testosterone (58 mCi/mmol) and dNTPs were purchased from Amersham Pharmacia Biotech, USA. [3H]-nitrosodimethylamine (NDMA) (54 mCi/mmol; 98% of purity) was synthesized and its purity checked as previously described (31). All other products were of the highest grade available.

**Human samples**

Human mucosa specimens were obtained from patients who were submitted to oesophagectomy or endoscopy. Patients with a confirmed diagnosis of squamous cell carcinoma were submitted to oesophagectomy at the Hospital das Clínicas, Porto Alegre, Rio Grande do Sul (four patients), or at Hospital Universitário Pedro Ernesto (HUPE–UERJ), Rio de Janeiro, Brazil. About 1 g of normal and 2 g of cancerous mucosa obtained from each patient were immediately frozen in liquid nitrogen, with fragments taken out for histopathologic analysis. Fragments of normal oesophageal mucosa were collected through endoscopy from 45 patients who attended at HUPE–UERJ, who were suffering from gastritis and had taken no medicine apart from anti-acids or non-steroidal anti-inflammatory drugs. The fragments were collected either from three distinct portions of the oesophagus (10 patients), or from the middle portion of the oesophagus (35 patients). A fragment of normal human liver was obtained from one patient attended at HUPE–UERJ who was submitted to liver biopsy to investigate liver jaundice. A fragment of normal breast tissue was obtained from a patient who attended at HUPE–UERJ who was submitted to reducing mammoplasty. Neither of them was taking any medication before donating the samples. Information was collected regarding smoking condition (number of cigarettes smoked/day/years of smoking), alcohol consumption (type of alcoholic beverage consumed and weekly frequency), age, colour, sex, and social–economic status. The Ethical Committee from Hospital das Clínicas–UFRGS, and HUPE–UERJ has approved the collection of samples as described by Hakkola et al. (33), and for proubiquitine as described by Sanguinetti et al. (54). The semi-quantitative comparison between the patients was done by measuring the intensities of the bands of the amplified fragments using a scanner linked to a Hitachi F-3010 spectrofluorimeter adjusted to 525 nm emission and excitation. For each patient there was one reverse transcription, and the peak areas for each amplified CYP and proubiquitine were calculated from three independent PCR reactions. For each patient, proubiquitine peak areas were calculated and the lowest value obtained among all patients was considered as 1 Unit of proubiquitine. The unit value of CYP expression of each patient was the mean ± SD of the three independent determinations normalised in relation to the areas obtained from the amplification of proubiquitine of the same patient. Both values were normalised in relation to 1 Unit of proubiquitine.

**Enzyme assays**

Oesophageal microsomes were prepared as previously described (23). Protein concentration was determined as described by Lowry et al. (35). Ethoxyresorufin-O-deethylase (EROD) assay was done as described by Burke et al. (36). The assays of ethoxycoumarin-0-deethylase (ECOD) and of coumarin-7-hydroxylase (COH) were done as described by Aflato (37), using 300 µM of substrate. Under these conditions, the limit of detection of umbelliferone was 2 pmol. The radiolabelled assay of [1-14C]-testosterone 6β-hydroxylase was done as described by Imaoka et al. (38), using 100 µM of substrate. The radiometric assay of [14C]-NDMA demethylase was done as described by Levin et al. (39), using 40 µM of NDMA. The incubation assays used 0.5 mg of microsomal protein and were carried out for 30 min. NDEA deethylase was done as previously described (23).

**Statistical analysis**

Statistical analysis was made by Student or Welch t-test using Instat 2.01 program (GraphPad Software, CA, USA).

**Results**

We analysed the expression of the main CYP enzymes capable of activating carcinogens in the oesophageal mucosa of 50 Brazilian patients. Both sexes were equally represented, and all patients were from low social–economic status, with most of them being white (66%). Among them, 32 were current or former smokers, with an average of 16 cigarettes smoked/day/19 years. Regarding alcohol consumption, 9% were heavy drinkers and 46% were light drinkers.

The expression of CYP enzymes was initially studied by RT-PCR, since for most samples, only endoscopic fragments were available. Figure 1 shows the results of a representative analysis of the middle portion of the oesophagus of 10 patients. CYP1A1 (Figure 1A), and CYP1A2 (Figure 1B) mRNA were not expressed in the oesophageal mucosa of any of the patients analysed. The presence of a 432 and a 308 bp fragments, corresponding to CYP1A1 (Figure 1A) and CYP1A2 (Figure 1B), respectively, amplified from reverse transcribed total RNA obtained from either breast or liver, served as positive controls. Figure 1C shows that CYP3A4 mRNA was not expressed in our oesophageal samples but only in liver as illustrated by the amplification of a 390 bp fragment. The presence of a 363 bp fragment amplified with primers for CYP2A6/2A7 (Figure 1D), however, shows that CYP2A6/2A7 mRNA is expressed in the oesophageal mucosa. From this representative gel, it can be clearly seen that there is a high degree of inter-individual variation in CYP2A6/2A7 mRNA expression levels among patients. Figure 1E shows the amplification of a 365 bp fragment, demonstrating the expression of CYP2E1 in the oesophageal mucosa. Although there is
CYP2A6/2A7 and CYP2E1 expression in oesophageal mucosa

Fig. 1. Expression of CYP 1A1 (A), CYP1A2 (B), CYP3A4 (C), CYP2A6/2A7 (D), CYP2E1 (E), and proubiquitine (F) mRNAs in the oesophageal mucosa, liver (L) and breast (B) of Brazilian patients.

Table I. Expression of CYP2A6/2A7 and CYP2E1 mRNA in the normal oesophageal mucosa of 50 Brazilian patients

<table>
<thead>
<tr>
<th>CYP expression</th>
<th>No. of patients (%)</th>
<th>Mean ± SDa</th>
<th>Range of valuesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A6/2A7</td>
<td>31 (61%)</td>
<td>0.61 ± 0.5</td>
<td>0.06–2.46</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>48 (96%)</td>
<td>0.73 ± 0.34</td>
<td>0.06–1.26</td>
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aValues expressed as normalised for 1 Unit of proubiquitine (see Materials and methods for details). Mean ± SD of CYP expression in the medium portion of the normal oesophageal mucosa of 50 patients.

bRange of values in CYP expression between the patients who had the lowest and the highest expression of the CYP mRNA.

Fig. 2. Expression of CYP2A6/2A7 (a), and CYP2E1 (b) mRNAs in proximal (P), medium (M), or distal (D) oesophageal mucosa of 10 patients.

some inter-individual difference in its expression, it does not seem to be as intense as for CYP2A6/2A7 (Figure 1D).

In order to evaluate the degree of inter-individual variation in the expression of these enzymes, we normalized their expression levels relative to that of proubiquitine. Table I shows that CYP2A6/2A7 mRNA was expressed in 61% of patients with a high variation in expression among those who expressed it. By comparison, CYP2E1 was expressed in almost all patients analysed (96%), with a lower, but comparable variation in expression. There was no correlation between smoking or drinking habits with the expression of CYP2A6/2A7 or CYP2E1.

Next, we analysed the regional expression of CYP2A6/2A7 and CYP2E1 mRNAs along three distinct portions of the oesophagus of 10 patients. Figure 2A shows that CYP2A6/2A7 was expressed in the distal portion of the oesophagus of nine patients, in the middle portion of the oesophagus of all 10 patients, but in the proximal portion of the oesophagus of only one patient (patient 7). It also shows that a high degree of variation in CYP2A6/2A7 levels occurs in patients who show expression in more than one portion of the oesophagus.

Figure 2B shows that CYP2E1 was expressed in the distal portion of the oesophagus of five patients, in the middle portion of the oesophagus of eight patients, but in the proximal portion of the oesophagus of only one patient (patient 10). When CYP2E1 was expressed at more than one site of the oesophagus of a patient, the expression level seemed to be rather uniform. CYP1A1, CYP1A2 and CYP3A4 mRNAs were not expressed in either the distal or proximal portions of the oesophagus of any of the patients (results not shown).

We also analysed CYP expression at the protein level in four patients from Rio Grande do Sul, and one from Rio de Janeiro, from whom we were able to obtain larger amounts of oesophageal mucosa through oesophagectomy. Table II shows that all of these five patients expressed CYP2E1 mRNA. We were also able to detect the demethylation of [14C]NDMA...
in all of the five patients analysed. A relatively low degree of inter-individual variation in activity was observed (18.2 ± 8.8 pmol/min/mg microsomal protein), in accordance with the degree of inter-individual difference in CYP2E1 mRNA expression. Furthermore, there was no significant difference between CYP2E1 mRNA expression and NDMAd activity of the normal mucosa and tumour of each patient. By contrast, the tumour but not the normal mucosa of one of these five patients expressed CYP2A6/2A7 mRNA, as shown in Table II. In fact, this patient presented the second highest expression of CYP2A6/2A7 mRNA among all of the patients analysed in this study. We detected the deethylation of NDEA with microsomes prepared from this patient’s tumour, but not from the corresponding normal mucosa or from any other patients’ samples. We also detected COH activity, a reaction catalysed by CYP2A6 (21), only in microsomes prepared from this same tumour. Since the level of CYP2E1 mRNA, or NDMA demethylation in this patient was not significantly different from the others, these results suggest that NDMA metabolism in this patient may be catalysed by CYP2A6. We could also detect ECOD, with an almost 5-fold higher activity of the normal mucosa and tumour of each patient. Finally, we could not detect the expression of CYP2E1 mRNA in nearly all patients analysed (96%), which is similar to the data obtained by Lechevrel et al. (27), who found that 90% of French patients expressed CYP2E1 mRNA in their oesophagus. Furthermore, whereas we detected a 21-fold variation in oesophageal CYP2E1 mRNA expression among Brazilian patients, Lechevrel et al. (27) detected a 41-fold variation in protein expression. These results suggest that there is a relatively high degree of inter-individual variation of CYP2E1 expression in the oesophagus. This is different from the liver, where it has been previously shown that the expression of CYP2E1 mRNA does not present high variability (40,41).

The expression of CYP2E1 mRNA and the presence of NDMAd in human oesophageag agree with previous results that show that NDMA can be metabolized by oesophageal cell explants (42). Furthermore, Smith et al. (28) showed recently that oesophageal microsomes prepared from American and Chinese patients were able to metabolize NDMA, with a smaller degree of inter-individual variation and an activity similar to what we observed in this study. However, the authors detected a lower NDMAd activity in tumour microsomes when compared with normal mucosa, which is in contrast with our results, which showed a slightly higher NDMAd activity in tumour microsomes.

CYP2A6/2A7 mRNA was expressed in the oesophagus of 61% of the patients analysed with an almost 2-fold higher degree of inter-individual variation than CYP2E1. CYP2A protein has been previously shown to be expressed in the oesophagus of French patients (27). A high variation in CYP2A6 expression was observed in the liver of Caucasian and Asian patients (43), and there seems to be a good correlation between CYP2A6 mRNA and protein expression (40). In this regard, only the tumour of one of the five patients from whom we had enough tissue to prepare microsomes expressed CYP2A6/2A7 mRNA, and microsomes prepared from it presented COH and NDEA deethylase activity, at levels similar to those of rat oesophageal microsomes (23). Since this patient’s tumour presented a high level of CYP2A6/2A7 mRNA expression, our results suggest that patients with high CYP2A6 mRNA oesophageal expression may be able to correlating localized CYP expression with tumour formation in different patients are necessary to support this association.

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activate certain carcinogens that are metabolized by CYP2A6. Although the primers used in this study are able to amplify CYP2A7, this enzyme seems to be expressed only in the liver (44), and when it was expressed in a heterologous system, CYP2A7 did not present any catalytic activity (45). CYP2A13 has recently been shown to be expressed at high levels in the human nasal mucosa (44,46), and when it was expressed in a heterologous system, CYP2A13 presented a higher activity in the metabolism of several nitrosamines than CYP2A6 (46). However, the primers used in this study do not amplify CYP2A13.

We did not detect CYP1A1, CYP1A2, and CYP3A4 expression in the oesophagus of any of the patients analysed. The findings reported in the literature concerning the expression of these enzymes in human oesophagus are conflicting. CYP3A4 protein, but not CYP1A1 or CYP2E1 has been detected at low levels in human oesophagus (47). CYP1A1 mRNA has been detected in the oesophagus of two out of six patients analysed, but only after omeprazole administration (33). Murray et al. (29) showed through immunoistochemistry that whereas CYP1A1 protein was expressed in normal mucosa of some patients, CYP1A1 and CYP3A were present in most of the cancerous oesophagus of Caucasian patients. Nakajima et al. (30) also detected the expression of CYP1A1 protein in 41 squamous cell carcinoma analysed from Chinese patients. CYP3A4 protein was also detected in some of them (30).

Recently, Lechevel et al. (27) detected the expression of CYP1A1 in most, and of CYP1A2 in some of the normal mucosa of the 25 French patients analysed. They also showed that CYP3A5, but not CYP3A4 was expressed in the oesophageal tissues of these patients. However, the primers used in our study do not amplify CYP3A5. Nevertheless, the absence of CYP1A1, CYP1A2 and CYP3A4 mRNA expression in our patients suggests major differences among different populations in the expression of oesophageal CYP enzymes. In addition, we did not detect EROD, MROD or testosterone 6β-hydroxylation in oesophageal microsomes, confirming the results obtained with RT–PCR analysis.

The rat is the most susceptible species for oesophageal experimental tumour induction (20), and it has been suggested that this is due to specific activation of nitrosamines by CYP enzymes. We have recently shown that CYP1A1 and CYP2A3, but not CYP2B1/2B2, CYP2E1, or CYP3A are expressed in the rat oesophagus (23). CYP2A3 is the rat orthologue of human CYP2A6 and of mouse CYP2A5, and these enzymes are excellent catalysts of the activation of nitrosamines such as NDEA (12,21–23), NNN (22,25), NMB2A (24), and NMAA (25). Therefore, the expression of CYP2A3 in the rat and of CYP2A6 in human oesophageus suggest that some individuals who express high levels of CYP2A6 in their oesophagus could be susceptible to the same nitrosamines that are activated in the rat oesophagus by CYP2A3.

A major difference between human and rat oesophageal CYP expression is that CYP2E1 is expressed in human, but not in rat oesophagus (23). This suggests that, as opposed to the rat, NDMA, which is activated exclusively by CYP2E1, could induce tumours in the human oesophagus. Furthermore, ethanol has been shown to inhibit nitrosamine metabolism in rat liver, but not oesophagus, increasing oesophageal exposure to nitrosamines (12–16) and, consequently, tumour formation (11). Our results suggest that if ethanol caused a hepatic disease, which decreased CYP expression in this tissue, it could change nitrosamine metabolism from liver to the oesophagus, as in the rat. Although ethanol could induce CYP2E1 expression, this would probably occur not only in the oesophagus, but also in the liver, where CYP2E1 expression and NDMA metabolism levels are much higher than those present in the human oesophagus.

We conclude that the oesophageal mucosa of Brazilian patients express CYP2A6 and CYP2E1, both enzymes being very active in nitrosamine activation. We are at the moment investigating if the human oesophagus expresses CYP2A7 and CYP2A13, analysing the polymorphism of CYP2A6 and CYP2E1 in oesophageal cancer patients, and correlating it to CYP expression and nitrosamine adduct levels to have a better understanding of the importance of the expression of these CYP enzymes in human oesophageus to oesophageal cancer.

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References


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