HEPATOBILIARY

DNA adducts, detected by $^{32}$P postlabelling, in human cholangiocarcinoma

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Background: Reported mortality from intrahepatic cholangiocarcinoma (CCa) has risen steeply in the UK and other industrialised countries over the past 30 years, the cause of which has not been explained. DNA adduct formation is promutagenic and demonstrates exposure to a DNA damaging agent. It is a key step in chemically induced carcinogenesis. We hypothesise that the increase in CCa mortality is caused by a rise in a genotoxic environmental agent(s), causing cholangiocyte DNA damage.

Aims: To investigate and compare tumour and tumour adjacent CCa tissue, and non-cancer control bile duct tissue, for DNA adducts as a biomarker of genotoxin exposure.

Methods: DNA from 32 intrahepatic CCa patients (and in 28 cases DNA from adjacent non-tumour tissue) and from biliary ducts of seven non-cancer patients were investigated for the presence of DNA adducts using the nuclease P1 method of $^{32}$P postlabelling. DNA adduct levels (number of adducts/10$^7$ nucleotides) were quantified.

Results: There was no significant difference in relative adduct labellings (RALs) between tumour adjacent DNA (median 8.6, range 1.2–51.6) and CCa DNA (7.2, 1.8–48.4). However, RALs were significantly higher in DNA from cancer patients (tumour adjacent and CCa DNA) compared with non-cancer patient DNA (2.9, 0.6–11.5; p=0.032, two tailed Mann-Whitney U test). Different adduct patterns were also seen in CCa compared with non-cancer patients.

Conclusion: Quantitative and qualitative differences in adducts between cancer and non-cancer patients support the hypothesis that genotoxins may play a role in the development of intrahepatic CCa.

Cholangiocarcinoma (CCa) is a malignant neoplasm arising from cholangiocytes in the epithelial lining of the biliary tree. It is usually fatal. Worldwide, CCa is the second commonest primary liver cancer after hepatocellular carcinoma, accounting for 15% of all primary hepatic malignancies. Overall, the incidence of CCa in Asia is 50 times higher than that in Europe where it has been regarded as a rare tumour. However, recent reports have shown that intrahepatic CCa (arising within the liver parenchyma) is steadily rising in industrialised countries (fig 1). Since the mid-1990s, CCa has become the commonest recorded cause of death from a malignant liver tumour in England and Wales.

The cause of the continuing rise in CCa in industrialised countries is unknown. It is not adequately explained by changes in mortality coding or improvements in diagnosis. Many carcinogenic toxins are metabolised via the hepatobiliary route and toxic compounds are linked to other primary liver tumours, for example alcohol and aflatoxin with hepatocellular carcinoma, and vinyl chloride with angiosarcomas.

The carcinogenic properties of such chemical toxins is likely to be mediated via genotoxic effects. One possible contributory factor to the rise in CCa may be the chronic and increasing exposure of cholangiocytes in the biliary ductal epithelium to environmental carcinogenic metabolites in bile.

DNA adducts

DNA adducts are covalently modified bases resulting from binding of electrophilic carcinogens, or their metabolites, at the nucleotide level. Adduct formation is a primary initiating event in genotoxic carcinogenesis and clearly demonstrates exposure to a DNA damaging agent. It is a key step in chemically induced carcinogenesis as mis- or unrepaired adducts can lead to mutation. As well as causing mutations, DNA adducts contribute to induction of carcinogenesis by chromosomal damage. The formation of DNA adducts in various human tissues has been extensively studied as a biomarker of exposure to occupational and other carcinogens in high risk populations. Most studies considering the association between cancer at different sites and adduct levels have shown that cancer cases have higher levels of DNA adducts.

Figure 1 Examples of countries where intrahepatic cholangiocarcinoma is rising, ASMR, age standardised mortality rate (modified from Khan and colleagues).

Abbreviations: CCa, cholangiocarcinoma; CYP, cytochrome P450; RAL, relative adduct labelling; TLC, thin layer chromatography.
of adducts than non-cancer controls.\textsuperscript{19} That bile contains chemicals with adduct inducing properties has been shown in a series of studies on patients with familial adenomatous polyposis\textsuperscript{20,21} in whom multiple adenomas in the large bowel and upper gastrointestinal tract undergo malignant transformation due to mutations in the APC tumour suppressor gene. Several studies have shown that DNA adducts are induced in human as well as rodent livers following exposure to carcinogenic chemicals. For example, a correlation was found between DNA adduct formation and mutagenic index from petroleum distillates.\textsuperscript{22} DNA adducts have also been detected in human liver cells following exposure to steroid hormones\textsuperscript{23} and polychlorinated biphenyls.\textsuperscript{24} Induction of DNA adducts has also been linked to the initiation of neoplasia leading to malignant change in haemochromatosis patients.\textsuperscript{25}

**Present study**

In over 200 studies, DNA adducts have been detected in various human tissues, including the liver, lung, colon, bladder, breast, cervix, brain, blood lymphocytes, and stomach.\textsuperscript{12} Currently, however, there are no published studies of DNA adduct detection in either malignant or normal human biliary tissue. Studies of the biliary system may be of considerable interest because of the role of the liver in the metabolism of xenobiotics and the concentration of such compounds in bile. In the present study, we compared DNA from CCa, tumour adjacent, and non-cancer control tissue, for levels and patterns of DNA adducts as a biomarker of genotoxin exposure.

Various methods exist for detecting DNA adducts in tissue, including accelerator mass spectrometry, fluorescence spectroscopy, gas chromatography/mass spectrometry, and immunoassay.\textsuperscript{10} We used \( ^{3}P \) postlabelling, one of the most sensitive and popular techniques. Its advantages include the ability to detect 1 adduct in \( 10^{10} \) normal nucleotides using as little as 1 \( \mu \)g of DNA. It is also unique in that it is a generic procedure requiring no prior adduct characterisation and therefore unknown adducts can be detected.\textsuperscript{12,26}

**MATERIALS AND METHODS**

**Collection of samples**

Tumour tissue samples were collected at surgery from 32 intrahepatic CCa patients (12 males, aged 36–70 years; mean 56) by hepatobiliary surgeons at Hammersmith Hospital, Imperial College, London. All samples were verified as primary intrahepatic cholangiocarcinomas by histology and radiology. In 28 cases, tumour adjacent tissue was also collected. Cystic ducts were collected from seven non-cancer patients (three males, aged 29–72 years; mean 54) undergoing laparoscopic cholecystectomy for gall stones. The cystic ducts were collected by hepatobiliary surgeons at St Mary’s Hospital, Imperial College, London. Local ethics committee approval was obtained for the present study.

**RESULTS**

There were qualitative differences, as well as significant quantitative differences, in adducts between cancer and non-cancer patients.

**Patterns of hot spots**

Examples of autoradiographs showing patterns of the major typical adduct spots that were obtained are shown in fig 2B–D, and a schematic diagram showing the positions of all spots obtained is shown in fig 2A. The numbers and spread of adduct spots are summarised in fig 3. There was no significant difference in the number of spots between tumour adjacent and CCa DNA—a median of four spots was seen in both groups. However, there were qualitative differences in the pattern and intensity of the spots between these two groups (fig 2B, C). Again, these differed from spots seen in DNA from control patients where three main areas of adducts were apparent (fig 2D). The positive control resulted in the typical well characterised adduct pattern expected from benzo (c) pyrene.
chrysene (data are available on request). The negative controls did not show adduct formation.

**Relative adduct labelling (RAL)**

There was no significant difference in RALs (number of adducts per 10^8 nucleotides) between tumour adjacent DNA (median 8.6, range 1.2–51.6) and CCa DNA (7.2, 1.8–48.4). However, RALs were significantly higher in DNA from cancer patients (tumour adjacent and CCa DNA) compared with non-cancer patient DNA (2.9, 0.6–11.5; p=0.032, two tailed Mann-Whitney U test). Individual RALs for the major typical adduct spots that were seen in each group are given in table 1. Comparisons of RALs between the three groups are shown in figs 4 and 5. There was no significant difference in total RALs between smokers and non-smokers in non-cancer subjects or cancer patients.

**DISCUSSION**

Intrahepatic cholangiocarcinoma has increased in industrialised countries over the past three decades. The reason for this rise remains unexplained. Given the relatively recent time scale of this increase, it is unlikely to be due to genetic changes in the populations involved, leaving the possibility of an environmental aetiological factor.

In parts of the globe where liver flukes are endemic, such as China and the north eastern provinces of Thailand, CCa is a common liver tumour. Humans are infected with the flukes...
Opisthorcis viverrini and Clonorchis sinensis) by eating insufficiently cooked fish containing the infective metacercariae. Chronic infection of mature flukes in the intrahepatic (and to a lesser extent in the extrahepatic) bile ducts causes epithelial dysplasia, a precancerous lesion which progresses to neoplastic transformation by an accumulation of molecular abnormalities activating proto-oncogenes and simultaneously inactivating tumour suppressor genes.1,2 In the Western world, in the absence of liver flukes, the actiology of most CCa are unknown. Although there are some conditions strongly associated with CCa (such as primary sclerosing cholangitis, ulcerative colitis, bile duct adenomas, biliary cysts, and chronic intraductal gall stones), in the vast majority of cases these risk factors do not apply and the cause is unknown.3,4

A possible explanation for the rise in intrahepatic, rather than extrahepatic, CCa may be due to the larger surface area of the intrahepatic biliary tree,5 allowing greater exposure to potential carcinogens, including natural and/or xenobiotic chemical residues in the environment, which are excreted and concentrated in bile. Several chemical agents have been implicated in the pathogenesis of pancreatic cancer6–10 and CCa11–13 including cadmium, organochlorines, asbestos, dioxins, ioniazid, methylxyp, nitrosamines, and polychlorinated biphenyls. Specific evidence of a chemical genotoxic carcinogen causing CCa comes from thorotrast.14,15 This colloidal suspension of thorium dioxide was used as a contrast agent before being banned in the 1960s due to its carcinogenic properties, particularly with regard to bile duct tumours. Its cancer causing effects are thought to be via induction of p53 mutations.16

DNA adducts were found in all samples tested. The concentrations of adducts we measured are similar to previous human adduct investigations using 32P postlabelling. These include studies of adduct inducing properties of bile in the gastrointestinal tract of patients with familial adenomatous polyposis17–20 (0–30 RALs), and studies of human liver exposed to chemical genotoxins21–23 (2–100 RALs). It is generally accepted that background levels of DNA adduction are essentially universal.24 Nevertheless, the finding of higher adduct levels in CCa compared with non-cancer patients in our study is consistent with the hypothesis that biliary cholangiocytes are exposed to DNA damaging agents and that the extent of DNA adduct formation, and subsequent mutation risk, correlates with the risk of tumorigenesis. Thus increasing adduct burden may lead to an increase in carcinogenic risk.25–27

Once formed, DNA adducts are usually removed by cellular excision repair mechanisms. Therefore, the presence of carcinogen-DNA adducts in tissues reflects a number of different processes, including exposure to toxin, toxin levels, and their metabolism to active species (or detoxification) depending on host enzymes, absorption, and DNA repair. The cytochrome P450 (CYP) family, for example, is particularly important for adduct formation resulting from the metabolism of polycyclic aromatic hydrocarbons (CYP2B1/2 and 2C9/10) and polychlorinated biphenyls (CYP-1A1, 1B1, CYP2B1/2).28–31 Hence DNA adduct levels provide a measure both of exposure and of host factors that affect these processes. Given that DNA adducts are widespread, but only a minority of people progress to develop cancer, presumably host factors, as well as exposure, play a key role in determining progress to cancerous change.

Pronounced inter-individual variations in the activity of enzymes that participate in the activation and inactivation pathways of carcinogen metabolism and DNA repair have been described.32 Such variation has been increasingly linked to differences in cancer susceptibility, potentially as a result of...
modifying DNA adduct levels. For example, a linear association has been observed between levels of bulky DNA adducts in human lung and expression of aryl hydrocarbon hydroxylase (CYP1A1). Also, in the lung, levels of bulky DNA adducts vary with glutathione S-transferase GSTP-1 genotype whereas N7- methyl guanine (the principle adduct arising from exogenous exposure to methylating agents) has been associated with different CYP2E1 and CYP2D6 genotypes.

DNA repair enzymes are important in determining adduct levels and subsequent cancer risk. In one study, O6-methyl guanine in bladder were inversely related to the DNA repair protein O6-alkyl-guanine-DNA alkyltransferase activity. Deficiency of repair systems increase carcinogenesis, for example skin cancer in xeroderma pigmentosa where UV light induced skin adducts are not correctly repaired.

Another factor that may modify the risk between adduct generation and subsequent carcinogenesis is variation in exposure to cell proliferating agents. Exposure to a DNA adduct inducing carcinogen on a background of increased chronic cholangiocyte turnover, for example secondary to liver fluke infestation, primary sclerosis cholangitis, or intraductal gall stones (all recognised risk factors for CCA), may provide the additional "hit" required for tumorigenesis.

The method of postlabelling we employed does not generally detect small endogenously arising adducts formed from interaction with, for example, methylating agents or free radicals. The adducts seen in this study are therefore likely to be due to exogenous agents but the possibility that some adducts may have originated endogenously cannot be completely excluded. However, the formation of endogenous, as well as exogenous, DNA adducts may be enhanced by environmental and/or host factors causing susceptibility to adduct formation in vivo.

Although postlabelling is a highly sensitive technique for the detection of unknown adducts where prior adduct characterisation is unavailable, it is not specific. The postlabelling method suffers from the disadvantage that adduct structure cannot be directly determined. Therefore, the origin and exact nature of these DNA modifications is unclear, given that there was no consistent history of exposure to therapeutic or occupational adduct forming agents in the patients studied. It must also be noted that not all DNA adducts are necessarily equal in terms of mutagenic potential. Different adducts are also likely to have differing half lives and propensity for repair. Furthermore, it must be borne in mind that adducts unique to tumours may not just represent a higher risk of carcinogenesis but theoretically may also reflect a neoplasia induced alteration in the metabolic profile of the cell, resulting in an altered capacity to metabolise carcinogens and thus form adducts. Hence it is difficult to form any conclusions as to the oncogenic significance of any individual adduct found. Large scale epidemiological investigations, including case control and clustering studies, may also yield important information as to any potential causative agents.

This study is the first to investigate DNA adducts in human bile duct cancer as a biomarker of carcinogen exposure. We found no significant difference in concentration or total number of adducts between CCA and adjacent non-tumour tissue, but found qualitative differences in the adduct patterns seen. We also found significantly more adducts in biliary tissue from patients with CCA compared with non-cancer individuals. Quantitative and qualitative differences in adducts between cancer and non-cancer patients support the hypothesis that genotoxins may play a role in the development of CCA. Further elucidation of causative factors will depend on epidemiological as well as laboratory studies.

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REFERENCES

1 Khan SA, Davidson BR, Goldin R, et al. Guidelines for the diagnosis and light induced skin adducts are not correctly repaired.


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