HEPATOBIILIARY

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/ 10^8 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.^{1–3} Worldwide, CCA is the second commonest primary liver cancer after hepatocellular carcinoma, accounting for 15% of all primary hepatic malignancies.² Overall, the incidence of CCA in Asia is 50 times higher than that in Europe where it has been regarded as a rare tumour.^{2–3} However, recent reports have shown that intrahepatic CCA (arising within the liver parenchyma) is steadily rising in industrialised countries (fig 1).^{4–6} Since the mid-1990s, CCA has become the commonest recorded cause of death from a malignant liver tumour in England and Wales.⁷

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.⁷ Many carcinogenic toxins are metabolised via the hepatobiliary route and toxic compounds are linked to other primary liver tumours, for example alcohol and aflatoxin⁸ with hepatocellular carcinoma, and vinyl chloride with angiosarcomas.⁹ The carcinogenic properties of such chemical toxins is likely to be mediated via genotoxic effects.^{8–9} 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.¹⁰ It is a key step in chemically induced carcinogenesis as mis- or unrepaired adducts can lead to mutation.^{10–12} As well as causing mutations, DNA adducts contribute to induction of carcino-

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

genesis 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.^{12–18} Most studies considering the association between cancer at different sites and adduct levels have shown that cancer cases have higher levels

Abbreviations: CCA, cholangiocarcinoma; CYP, cytochrome P450; RAL, relative adduct labelling; TLC, thin layer chromatography.

of adducts than non-cancer controls.¹⁹ That bile contains chemicals with adduct inducing properties has been shown in a series of studies on patients with familial adenomatous polyposis^{20–24} 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.²⁵ DNA adducts have also been detected in human liver cells following exposure to steroid hormones²⁶ and polychlorinated biphenyls.²⁷ Induction of DNA adducts has also been linked to the initiation of neoplasia leading to malignant change in haemachromatosis patients.²⁸

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.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.¹⁰ We used ³²P postlabelling, one of the most sensitive and popular techniques. Its advantages include the ability to detect 1 adduct in 10¹⁰ normal nucleotides using as little as 1 µg 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.^{12–29}

MATERIALS AND METHODS

Collection of samples

Tumour tissue samples were collected at surgery from 32 intra-hepatic 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 intra-hepatic 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 sample collection. Tissue samples were stored at –70°C prior to DNA extraction. Phenol extraction of DNA from tissue samples was carried out using a modification of the method devised by Gupta.³⁰ For postlabelling, DNA from mouse skin exposed to benzo (c) chrysene, known for its marked ability to form DNA adducts, and salmon sperm DNA were used as positive and negative controls, respectively. Three of the non-cancer patients (43%) and six of the CCa patients (19%) were smokers.

Reagents

Micrococcal nuclease, spleen phosphodiesterase, and nuclease P1 were obtained from Sigma (Poole, Dorset, UK), and T4 polynucleotide kinase from Gibco/BRL (Life Technologies, Paisley, UK). γ -³²P-ATP was obtained from ICN (Thame, Oxfordshire, UK). PEI-cellulose plates were manufactured by Macherey-Nagel. Salmon sperm DNA was obtained from Sigma, as were RNase and phenol for DNA extraction.

³²P postlabelling assays

Digestion and ³²P postlabelling assays

The concentration and purity of DNA extracted from tissue samples were determined by A260 and A280 ultraviolet spectroscopy readings using a Shimadzu MPS-2000 spectrometer. Samples of DNA (4 µg) were evaporated to dryness in a Gyrovap rotary drier and incubated overnight at 37°C with 0.12 U of micrococcal nuclease and spleen phosphodiesterase (1 µg/µl), 0.8 µl of calcium chloride/sodium succinate (50 mM CaCl₂ and 100 mM sodium succinate, pH 6.0), and 2.8 µl of distilled H₂O. The following day samples were incubated at 37°C for one hour with 0.96 µl of nuclease P1 (1.25 µg/µl), 1.44 µl of zinc chloride (2 mM), and 2.4 µl of sodium acetate (0.25 M, pH 5.0). Then, 1.92 µl of 0.5 M Tris base was added to each sample before labelling with 50 µCi of γ -³²P-ATP (specific activity 7000 Ci/mmol), 1 µl of kinase buffer (200 mM bicine, 100 mM MgCl₂, 100 mM dithiothreitol, 10 mM spermidine, pH 9.0), and 6 U of T4-polynucleotide kinase (0.6 U/µl, final concentration). Samples were incubated for 60 minutes at 37°C.

Thin layer chromatography (TLC)

Samples were spotted onto 10×10 cm polyethyleneimine-cellulose TLC plates and developed in solvent D1 (1 M sodium phosphate, pH 6.0) overnight onto a wick. The wicks were discarded, and the plates washed in D2 buffer (2.5 M ammonium formate, pH 3.5). Next, the plates were turned through 180° and developed in D3 solvent (3.5 M lithium formate and 8.5 M urea, pH 3.5). Plates were then turned through 90° and developed in D4 solvent (0.8 M lithium chloride, 0.5 M Tris HCl, and 8.5 M urea, pH 8.0). Both D3 and D4 were run to the top of the plates.

Detection, quantitation, and statistical analysis

Adduct spots were detected by autoradiography. Plates were exposed to Biomax-MS film (Kodak) overnight at –70°C. Scintillation readings were taken from hot spots representing adducts and blank areas from each plate using a Minaxi Tri-Carb 4000 series counter. For every sample relative adduct labelling (RAL) values (number of adducts per 10⁸ nucleotides) were determined in two separate assays, performed independently, to ensure consistency in the technique. RALs were calculated from the ratio of counts per minute to (specific activity×quantity of DNA labelled), as has been previously described.³¹ RALs in the second assays were reproducible to within 12% of the first RAL score. The adduct patterns were also reproducible on duplicate assays. As the data did not conform to a normal distribution (Shapiro-Wilk test 0.010), differences between groups were tested for significance using the Mann-Whitney U test.

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)

Figure 2 (A) Schematic diagram showing the position of all spots found, and solvent directions. A1–A3: spots seen in non-cancer DNA and common to most cholangiocarcinoma patients also, including tumour and tumour adjacent tissue. All three are shown in (D). C1–C4: spots seen in cholangiocarcinoma DNA only. Examples of C1–C3 are shown in (C). T1–T5, spots seen in tumour adjacent DNA only. Examples of T2, T4, and T5 are shown in (B). Arrows represent the solvent directions performed for D3 and D4. Various combinations of A1–A3 and C1–C4, or A1–A3 and T1–T5 were seen. (B–D) Examples of autoradiographs of ³²P postlabelled DNA (the origin of each autoradiograph is in the bottom left hand corner).

Figure 3 Frequency of numbers of adduct spots in (A) tumour adjacent DNA, (B) cholangiocarcinoma DNA, and (C) non-cancer control DNA, showing that the pattern of spots was similar in tumour and tumour adjacent tissue DNA but different to non-cancer DNA.

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 10⁸ 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.⁴ 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.

Table 1 Individual relative adduct labelling values (RALs, number of adducts per 10⁸ nucleotides) for the major typical adducts seen in each group of DNA samples, with group medians (range) of total RALs*

Median (range) of total RALs in each group	Major typical adducts	Individual RAL
Non-cancer DNA 2.9 (0.6–11.5)	A1	2.9
	A2	8.0
	A3	0.6
Cholangiocarcinoma DNA 7.2 (1.8–48.4)	C1	7.2
	C2	14.7
	C3	19.6
Tumour adjacent DNA 8.6 (1.2–51.6)	T2	8.6
	T4	16.2
	T5	22.5

*A table listing the entire data set: median RALs and distributions of all adducts (A1–3, C1–4, and T1–5) in cholangiocarcinoma, tumour adjacent, and non-cancer DNA samples, is available from the authors on request.

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.³² Humans are infected with the flukes

Figure 4 Distribution of relative adduct labelling (RAL) values (number of adducts per 10⁸ nucleotides) in (A) tumour adjacent DNA, (B) cholangiocarcinoma DNA, and (C) non-cancer control DNA.

Figure 5 Relative adduct labelling values (RALs; number of adducts per 10⁸ nucleotides) in the three sample groups. Group 1, tumour adjacent DNA (n=28); group 2, cholangiocarcinoma DNA (n=32); and group 3, non-cancer DNA (n=7).

(*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.^{2 3} In the Western world, in the absence of liver flukes, the aetiology 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.^{2 3 33}

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,³⁴ 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 cancer^{35 36} and

CCA,^{2 3 33 37–39} including cadmium, organochlorines, asbestos, dioxins, isoniazid, methyldopa, nitrosamines, and polychlorinated biphenyls. Specific evidence of a chemical genotoxic carcinogen causing CCa comes from thorotrast.^{38 39} 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.⁴⁰

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 polyposis^{20–24} (0–30 RALs), and studies of human liver exposed to chemical genotoxins^{26 27} (2–100 RALs). It is generally accepted that background levels of DNA adduction are essentially universal.⁴¹ 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.^{11 12 41}

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).^{42 43} 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.⁴⁴ Such variation has been increasingly linked to differences in cancer susceptibility, potentially as a result of

modifying DNA adduct levels.¹² For example, a linear association has been observed between levels of bulky DNA adducts in human lung and expression of aryl hydrocarbon hydroxylase (CYP1A1).⁴⁵ 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.⁴⁶

DNA repair enzymes are important in determining adduct levels and subsequent cancer risk. In one study, O⁶-N7-methyl guanine levels in bladder were inversely related to the DNA repair protein O⁶-alkyl-guanine-DNA alkyltransferase activity.⁴⁷ 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 sclerosing 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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