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ORIGINAL ARTICLE

Dynamic transcriptional regulatory complexes including BORIS, CTCF and Sp1 modulate NY-ESO-1 expression in lung cancer cells

Y Kang, JA Hong, GA Chen, DM Nguyen and DS Schrump

Thoracic Oncology Section, Surgery Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA

Previously, we reported that the paralogous zinc-finger proteins – CTCF and brother of the regulator of imprinted sites (BORIS), directly contribute to transcriptional regulation of *NY-ESO-1* in lung cancer cells. To further examine mechanisms that mediate expression of this cancer-testis gene, we performed software-guided analysis of the *NY-ESO-1* promoter region, which revealed several potential Sp1-binding motifs. Sequential 5-aza-2′deoxycytidine/depsipeptide FK228 treatment markedly induced BORIS expression and enhanced nuclear translocation of Sp1 in lung cancer cells. Transient transfection assays using promoter–reporter constructs, as well as gel-shift and chromatin immunoprecipitation experiments revealed that *NY-ESO-1* promoter activity coincided with occupancy of the proximal Sp1-binding site in lung cancer cells. Mutations within the Sp1 recognition sequence specifically eliminated binding of Sp1 to this motif *in vitro*, and markedly diminished *NY-ESO-1* promoter activity *in vivo*. siRNA-mediated inhibition of *Sp1* expression decreased *NY-ESO-1* promoter activity, whereas knock down of *CTCF* expression augmented *NY-ESO-1* transcription in lung cancer cells. Co-immunoprecipitation experiments indicated that Sp1 physically interacts with BORIS but not with CTCF *in vivo*. Collectively, these findings suggest that BORIS recruits Sp1 to mediate de-repression of *NY-ESO-1* during pulmonary carcinogenesis.
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Keywords: lung cancer; epigenetics; NY-ESO-1; BORIS; CTCF; Sp1

Introduction

Cancer-testis antigens (CTA) constitute a unique and growing class of germ cell-related proteins that are aberrantly expressed in a variety of human malignancies, and are recognized by cytolytic T lymphocytes (CTL) from cancer patients (Simpson *et al.*, 2005). During recent years, the CTAs have emerged as attractive targets for cancer immunotherapy. Of particular interest in this regard, are CTAs encoded on the X chromosome (CT-X), such as NY-ESO-1, which elicits cellular, as well as humoral immunity in approximately 50% of patients whose tumors express this cytoplasmic protein (Gnjatic *et al.*, 2006).

Recent studies indicate that the nuclear proteins CTCF and BORIS (Brother of the Regulator of Imprinted Sites) may contribute to regulation of *NY-ESO-1* expression during pulmonary carcinogenesis (Hong *et al.*, 2005). CTCF is a ubiquitous DNA-binding protein bearing a central 11 zinc (Zn)-finger region that contributes to formation of chromatin insulator/boundaries, X chromosome inactivation, reading of gene-imprinting marks, and regulation of a variety of genes mediating cell cycle progression and apoptosis (reviewed by Ohlsson *et al.* (2001); Klenova *et al.* (2002)). BORIS exhibits considerable homology to CTCF in the central 11 Zn-finger DNA-binding domain, but not in the NH_2- and COOH-terminal regions that constitute approximately two-thirds of the full-length amino-acid sequences of these proteins (Loukinov *et al.*, 2002). Thus, whereas CTCF and BORIS apparently recognize the same DNA-binding sequences, these transcription factors may exhibit significant differences regarding recruitment of functional partners (reviewed in Klenova *et al.* (2002)). Unlike *CTCF*, *BORIS* is not expressed in normal somatic cells (Klenova *et al.*, 2002; Loukinov *et al.*, 2002). In male germ cells, transient expression of *BORIS* coincides with a marked decrease in *CTCF* expression, erasure of global DNA methylation patterns, and upregulation of CT genes (Loukinov *et al.*, 2002).

Recently, we reported that *BORIS* is activated during pulmonary carcinogenesis, and that *BORIS* expression coincides with de-repression of *NY-ESO-1* (Hong *et al.*, 2005). In addition, we have demonstrated that *BORIS* as well as *NY-ESO-1* expression can be induced in cultured lung cancer cells (but not normal human bronchial epithelial (NHBE) cells) following exposure to the DNA-demethylating agent 5-aza-2′ deoxycytidine (DAC), the histone deacetylase (HDAC) inhibitor Depsipeptide FK228 (DP), or sequential DAC/DP. Furthermore, we have shown that a CTCF-to-BORIS-shift in occupancy of the *NY-ESO-1* promoter coincides with de-repression of this CT gene in lung cancer cells

Correspondence: Dr DS Schrump, Thoracic Oncology Section, Surgery Branch, Center for Cancer Research, National Cancer Institute, Building 10, Rm 4-3942, 10 Center Drive, Bethesda, MD 20892-1201, USA.
E-mail: David_Schrump@nih.gov
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Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55 000 vascular deaths

*Prospective Studies Collaboration**

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*Collaborators listed in full at end of paper

Correspondence to:
PSC secretariat, Clinical Trial Service Unit and Epidemiological Studies Unit (CTSU), Richard Doll Building, University of Oxford, Oxford OX3 7LF, UK
psc@ctsu.ox.ac.uk

Summary

Background Age, sex, and blood pressure could modify the associations of total cholesterol (and its main two fractions, HDL and LDL cholesterol) with vascular mortality. This meta-analysis combined prospective studies of vascular mortality that recorded both blood pressure and total cholesterol at baseline, to determine the joint relevance of these two risk factors.

Methods Information was obtained from 61 prospective observational studies, mostly in western Europe or North America, consisting of almost 900 000 adults without previous disease and with baseline measurements of total cholesterol and blood pressure. During nearly 12 million person years at risk between the ages of 40 and 89 years, there were more than 55 000 vascular deaths (34 000 ischaemic heart disease [IHD], 12 000 stroke, 10 000 other). Information about HDL cholesterol was available for 150 000 participants, among whom there were 5000 vascular deaths (3000 IHD, 1000 stroke, 1000 other). Reported associations are with usual cholesterol levels (ie, corrected for the regression dilution bias).

Findings 1 mmol/L lower total cholesterol was associated with about a half (hazard ratio 0·44 [95% CI 0·42–0·48]), a third (0·66 [0·65–0·68]), and a sixth (0·83 [0·81–0·85]) lower IHD mortality in both sexes at ages 40–49, 50–69, and 70–89 years, respectively, throughout the main range of cholesterol in most developed countries, with no apparent threshold. The proportional risk reduction decreased with increasing blood pressure, since the absolute effects of cholesterol and blood pressure were approximately additive. Of various simple indices involving HDL cholesterol, the ratio total/HDL cholesterol was the strongest predictor of IHD mortality (40% more informative than non-HDL cholesterol and more than twice as informative as total cholesterol). Total cholesterol was weakly positively related to ischaemic and total stroke mortality in early middle age (40–59 years), but this finding could be largely or wholly accounted for by the association of cholesterol with blood pressure. Moreover, a positive relation was seen only in middle age and only in those with below-average blood pressure; at older ages (70–89 years) and, particularly, for those with systolic blood pressure over about 145 mm Hg, total cholesterol was negatively related to haemorrhagic and total stroke mortality. The results for other vascular mortality were intermediate between those for IHD and stroke.

Interpretation Total cholesterol was positively associated with IHD mortality in both middle and old age and at all blood pressure levels. The absence of an independent positive association of cholesterol with stroke mortality, especially at older ages or higher blood pressures, is unexplained, and invites further research. Nevertheless, there is conclusive evidence from randomised trials that statins substantially reduce not only coronary event rates but also total stroke rates in patients with a wide range of ages and blood pressures.

Introduction

The effects of other vascular risk factors—particularly blood pressure—on the epidemiological associations of cholesterol with ischaemic heart disease (IHD) and stroke remain uncertain. Although blood levels of total cholesterol are used widely to predict IHD, the relative risk per unit change in cholesterol decreases with age[1,2] and, perhaps, blood pressure,[3,4] and it is unclear whether an importantly positive association persists into old age. Furthermore, total cholesterol consists largely of the cholesterol in low-density lipoprotein particles (LDL cholesterol) plus the cholesterol in high-density lipoprotein particles (HDL cholesterol), which have opposite associations with IHD risk. Results from randomised trials have shown that treatment with a statin, which lowers LDL cholesterol, substantially reduces the incidence of IHD.[5] These trials have also shown a substantial reduction in the incidence of ischaemic stroke (without any apparent increase in haemorrhagic stroke).[5] The definite reduction in total stroke in the statin trials contrasts strongly with the weakness of the epidemiological association between blood cholesterol and stroke,[1,6–13] and that epidemiological association needs further exploration.

The results from retrospective epidemiological studies of IHD or stroke can be distorted by reverse causality (since vascular disease can itself directly or indirectly affect both blood cholesterol and blood pressure). In people with no previous history of vascular disease, however, prospective epidemiological studies have to be very large to assess reliably the extent to which one risk factor affects the relevance of another. The Prospective Studies Collab-

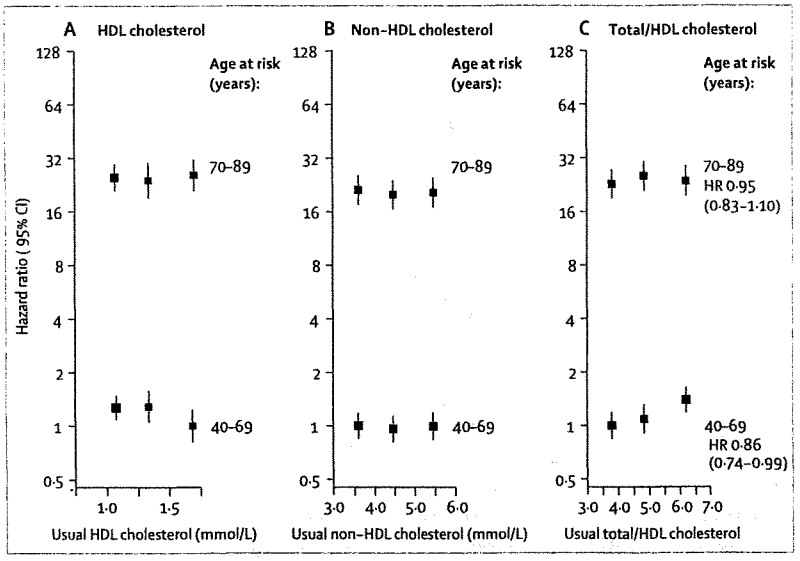

Figure 6: **Stroke mortality (914 deaths) versus usual (A) HDL cholesterol; (B) non-HDL cholesterol; and (C) total/HDL cholesterol**

Age-specific associations. Conventions as in figures 1 and 3. HR denotes the hazard ratio per 1·33 lower total/HDL cholesterol.

231 to haemorrhagic stroke, and 61 to subarachnoid haemorrhage. No material differences between the results for particular stroke subtypes were apparent (data not shown), but the numbers of deaths are too small for these findings to be informative.

Many of the vascular deaths that were not attributed to IHD or stroke may in fact have been directly or indirectly due to one or both of these diseases, and for the aggregate of all other vascular deaths the relative risks in each age range were intermediate between those for IHD and stroke. Taking the combined data from PSC and MRFIT, total cholesterol was positively associated with other vascular mortality in every 10-year age range up to age 80 years, but not at ages 80–89 years (table 2). Further details are shown in webfigures 9–11. A slightly J-shaped association before 70 years of age (webfigure 9) almost disappeared after excluding the first 5 years of follow-up (data not shown), suggesting that it may have been largely or wholly due to reverse causality. Further adjustment for smoking and systolic blood pressure did not materially alter the shapes or strengths of these associations. Parallel analyses of the MRFIT study yielded somewhat stronger associations, but again did not materially alter the age-specific findings (webfigure 10). In men, other vascular mortality was positively associated with total cholesterol in each age range (and the association was highly significant in every range except 80–89 years). In women older than 60 years, however, no positive association was apparent, despite the strongly positive association of female IHD mortality with total cholesterol (webfigure 3). In participants with data for HDL cholesterol, there were 1032 deaths from vascular causes other than IHD or stroke, and there was a positive association with the ratio total/HDL cholesterol that appeared to be steeper in middle age than in old age (webfigure 11).

For the aggregate of all non-vascular causes of death there was a negative association between total cholesterol and mortality (HR 1·10 [1·08–1·11] per 1 mmol/L lower total cholesterol; 42 865 deaths at ages 40–89 years). However, this negative association might be largely or wholly non-causal (eg, a consequence of confounding or of specific non-vascular diseases lowering total cholesterol), since it is weakened when the first 5 years of follow-up are excluded (data not shown), and the randomised trials of statins (which substantially lower total cholesterol) show no adverse effect on cancer or other non-vascular mortality.[5]

For both IHD and stroke, there was statistically significant heterogeneity between the HRs for individual studies (both $p<0·00001$) that could not be accounted for by differences in age, sex, smoking, the type of blood sample collected (fasting or non-fasting; serum or plasma), the method of outcome ascertainment, or the year of baseline survey (data not shown). There was a small trend for the age-specific associations of baseline cholesterol with mortality to be weaker in the studies with longer follow-up and in those that had relied on death certificates alone to code deaths, but allowance for this trend did not materially reduce the heterogeneity. There was, however, little evidence that extreme results from a few atypical studies had appreciably biased the overall results—after excluding successively the studies that contributed most to the heterogeneity (until, for IHD and for stroke, the p values for heterogeneity in the remaining studies were greater than 0·1), the estimated HRs were unaltered to two decimal places.

Discussion

This collaborative meta-analysis of almost 900 000 individuals in 61 prospective observational studies, with 55 000 vascular deaths during nearly 12 million person-years of follow-up, has characterised reliably the age-specific associations of total cholesterol with IHD, stroke, and other vascular mortality, and has assessed the quantitative and qualitative relevance of other risk factors to these associations. For IHD mortality, age and blood pressure substantially affected the strength of the proportional difference in risk associated with a specific difference in cholesterol, but did not affect its direction. For stroke mortality, however, age and blood pressure affected not only the strength but also the direction of the association. (Results for other vascular mortality are intermediate between those for IHD and stroke.) This collaboration has also, on the basis of only 3000 IHD deaths, assessed the independent relevance of HDL cholesterol, showing that the ratio of total to HDL cholesterol is a substantially more informative predictor of IHD mortality than are total cholesterol, HDL cholesterol, or non-HDL cholesterol.

Age substantially attenuates the proportional relation of IHD mortality with cholesterol (table 2, figure 1). However, total cholesterol is a strongly positive risk factor for IHD mortality throughout the main range of measured cholesterol values (about 3·5–9·5 mmol/L, which corresponds to a range of usual cholesterol values of about 4–8 mmol/L) not only in early middle age (when each mmol/L lower usual total cholesterol is associated with a halving of IHD mortality) but also in old age (when it is associated with a sixth lower IHD mortality). Little of the strength of the relation with IHD mortality (table 2) can be accounted for by the association of cholesterol with systolic blood pressure, since a difference of 1 mmol/L in usual cholesterol was associated with a difference of only about 2 mm Hg systolic blood pressure. At ages 60–69 years, for example, 2 mm Hg lower usual systolic blood pressure would be associated with an IHD HR of about 0·94,[15] and allowance for this systolic blood pressure would change the IHD HR of 0·68 in table 2 into 0·72 (ie, 0·68/0·94).

Although the proportional differences in risk decrease with age, the absolute effects of cholesterol on annual IHD mortality rates are much greater at older than at younger ages (see figure 1). For example, the absolute difference in the annual risk of IHD death for a 1 mmol/L difference in total cholesterol was about ten times greater at 80–89 years than at 40–49 years of age. Furthermore, the absolute effects at a specific age were somewhat greater for smokers than for non-smokers, and somewhat greater for obese than for non-obese individuals (since the hazard ratios for these factors were approximately multiplicative with those for cholesterol). At a specific age, however, the absolute effects on IHD mortality of cholesterol and of blood pressure were approximately independent of each other (ie, the absolute effects of cholesterol and blood pressure were roughly additive rather than multiplicative), so blood pressure somewhat attenuated the proportional effects of blood cholesterol on IHD mortality.

HDL cholesterol added greatly to the predictive ability of total cholesterol. (This result differs from the findings of a smaller meta-analysis[20] which suggested no additional advantage in measuring HDL cholesterol.) Higher HDL cholesterol and lower non-HDL cholesterol levels were approximately independently associated with lower IHD mortality, so the ratio of total/HDL cholesterol was substantially more informative about IHD mortality than either, and was more than twice as informative as total cholesterol. Because higher non-HDL cholesterol levels predicted similar relative risks at both above-average and below-average HDL cholesterol levels, the absolute relevance of LDL cholesterol is likely to be greater if HDL cholesterol levels are low. Other lipid-related measurements (eg, of apo B and apo A,[21,22] or of small dense LDL cholesterol particles[22]) may add more predictive power, and more detailed measurements of lipoprotein particles of many different sizes or types could well prove even more informative than any of these measurements.

Although haemorrhage accounts for a substantial proportion of fatal strokes (table 1), for many of the strokes in these studies the type (haemorrhagic or ischaemic) was not verified by a CT or MRI scan or by any other reliable method. Hence, misclassification may have attenuated any real associations with specific types of stroke, particularly at older ages when death certificates become less reliable. (Since all analyses are standardised for age, the relative risks in old age should not be materially biased by selective mortality at earlier ages.) Nevertheless, total stroke mortality and ischaemic stroke mortality in the present analyses were positively associated with total cholesterol only in middle age (table 2, figure 4) and only in those with lower blood pressure (baseline systolic blood pressure less than about 145 mm Hg; figure 5). Moreover, even in middle age, the positive association with stroke mortality was not strong (mortality ratio of 0·93 at ages 60–69 years: table 2), and can be approximately accounted for by the association of each 1 mmol/L usual total cholesterol with about 2 mm Hg systolic blood pressure (since 2 mm Hg lower usual systolic blood pressure would be associated with a stroke hazard ratio of about 0·92 at ages 60–69 years).[15] Even before allowance for systolic blood pressure, total cholesterol was not positively associated with ischaemic stroke mortality at older ages (or at higher levels of blood pressure), and was negatively associated with haemorrhagic and with total stroke mortality in these subgroups. Other observational studies have also suggested that total cholesterol is negatively associated with haemorrhagic stroke in people with high blood pressure.[3,8,23]

By contrast, meta-analyses of the randomised trials of just a few years of statin therapy to lower cholesterol, which greatly reduces the number of circulating LDL particles, have shown that regimens that reduce LDL cholesterol by about 1·5 mmol/L reduce by about a third the incidence not only of IHD but also of ischaemic stroke, approximately independently of age, blood pressure, or prerandomisation blood lipid concentrations (while appearing not to increase the incidence of haemorrhagic stroke).[5] The contrast between the statistically reliable results from randomised trials[5] for stroke and the present statistically reliable observational epidemiological results for stroke is substantial. Further investigation of exactly how lipoprotein particles affect stroke risks might help to explain this striking discrepancy.

Randomised trials of cholesterol-lowering statin therapy in a wide range of patient populations have shown substantial reductions in the incidence of IHD and of stroke.[5,24] In the PSC, the continuous positive relations observed at all ages between total cholesterol and IHD mortality, irrespective of the level of blood pressure, are in keeping with these randomised trial results, and with strategies to lower population levels of LDL cholesterol in all age groups.[25] The absence of any

independently positive association between total cholesterol and stroke mortality in middle age (after allowing for systolic blood pressure) or in those with systolic blood pressure below 145 mm Hg, and the negative association of cholesterol with stroke mortality at older ages or at higher blood pressures, are unexplained, and invite research. Irrespective of the explanation, however, treatment should be guided principally by the definitive evidence from randomised trials,[5] that statins substantially reduce not only coronary event rates but also total stroke rates in patients with a wide range of ages and blood pressures.

Contributors

All members of the writing committee contributed to the collection and analysis of the data, and to the preparation of the report. All collaborators had an opportunity to contribute to the interpretation of the results and to the redrafting of the report. The writing committee accepts full responsibility for the content of this paper.

Membership of the Prospective Studies Collaboration

Writing Committee—Sarah Lewington, Gary Whitlock, Robert Clarke, Paul Sherliker, Jonathan Emberson, Jim Halsey, Nawab Qizilbash, Richard Peto, Rory Collins.
Steering Committee—S Lewington (coordinator and statistician), S MacMahon (chair), R Peto (statistician), A Aromaa, C Baigent, J Carstensen, Z Chen, R Clarke, R Collins, S Duffy, D Kromhout, J Neaton, N Qizilbash, A Rodgers, S Tominaga, S Törnberg, H Tunstall-Pedoe, G Whitlock.
PSC Collaborators—Atherosclerosis Risk in Communities (ARIC): L Chambless; *Belgian Inter-university Research on Nutrition and Health (BIRNH):* G De Backer, D De Bacquer, M Kornitzer; *British Regional Heart Study (BRHS):* P Whincup, SG Wannamethee, R Morris; *British United Provident Association (BUPA):* N Wald, J Morris, M Law; *Busselton:* M Knuiman, H Bartholomew; *Caerphilly and Speedwell:* G Davey Smith, P Sweetnam, P Elwood, J Yarnell; *Cardiovascular Health Study (CHS):* R Kronmal; *CB Project:* D Kromhout; *Charleston:* S Sutherland, J Keil; *Copenhagen City Heart Study:* G Jensen, P Schnohr; *Evans County:* C Hames (deceased), A Tyroler; *Finnish Mobile Clinic Survey (FMCS):* A Aromaa, P Knekt, A Reunanen; *Finrisk:* J Tuomilehto, P Jousilahti, E Vartiainen, P Puska; *Flemish Study on Environment, Genes and Health (FLEMENGHO):* T Kuznetsova, T Richart, J Staessen, L Thijs; *Research Centre for Prevention and Health (Glostrup Population Studies):* T Jorgensen, T Thomsen; *Honolulu Heart Program:* D Sharp, J D Curb; *Imperial College and Oxon Clinical Epidemiology Limited:* N Qizilbash; *Ikawa, Noichi and Kyowa:* H Iso, S Sato, A Kitamura, Y Naito; *Centre d'Investigations Preventives et Cliniques (IPC), Paris:* A Benetos, L Guize; *Israeli Ischaemic Heart Disease Study:* U Goldbourt; *Japan Railways:* M Tomita, Y Nishimoto, T Murayama; *Lipid Research Clinics Follow-up Study (LRC):* M Criqui, C Davis; *Midspan Collaborative Study:* C Hart, G Davey-Smith, D Hole, C Gillis; *Minnesota Heart Health Project (MHHP) and Minnesota Heart Survey (MHS):* D Jacobs, H Blackburn, R Luepker; *Multiple Risk Factor Intervention Trial (MRFIT):* J Neaton, L Eberly; *First National Health and Nutrition Examination Survey Epidemiologic Follow-up Study (NHEFS):* C Cox; *NHLBI Framingham Heart Study:* D Levy, R D'Agostino, H Silbershatz; *Norwegian Counties Study:* A Tverdal, R Selmer; *Northwick Park Heart Study (NPHS):* T Meade, K Garrow, J Cooper; *Nurses' Health Study:* F Speizer, M Stampfer; *Occupational Groups (OG), Rome:* A Menotti, A Spagnolo; *Ohasama:* I Tsuji, Y Imai, T Ohkubo, S Hisamichi; *Oslo:* L Haheim, I Holme, I Hjermann, P Leren; *Paris Prospective Study:* P Ducimetiere, J Empana; *Perth:* K Jamrozik, R Broadhurst; *Prospective Cardiovascular Munster Study (PROCAM):* G Assmann, H Schulte; *Prospective Study of Women in Gothenburg:* C Bengtsson, C Björkelund, L Lissner; *Puerto Rico Health Heart Program (PRHHP):* P Sorlie, M Garcia-Palmieri; *Rancho Bernado:* E Barrett-Connor, M Criqui, R Langer; *Renfrew and Paisley study:* C Hart, G Davey Smith, D Hole; *Saitama Cohort Study:* K Nakachi, K Imai; *Seven Cities China:* X Fang, S Li; *Seven Countries (SC) Croatia:* R Buzina; *SC Finland:* A Nissinen; *SC Greece (Greek Islands Study):* C Aravanis, A Dontas, A Kafatos; *SC Italy:* A Menotti; *SC Japan:* H Adachi, H Toshima, T Imaizumi; *SC Netherlands:* D Kromhout; *SC Serbia:* S Nedeljkovic, M Ostojic; *Shanghai:* Z Chen; *Scottish Heart Health Study (SHHS):* H Tunstall-Pedoe; *Shibata:* T Nakayama, N Yoshiike, T Yokoyama, C Date, H Tanaka; *Tecumseh:* J Keller; *Tromso:* K Bonaa, E Arnesen; *United Kingdom Heart Disease Prevention Project (UKHDPP):* H Tunstall-Pedoe; *US Health Professionals Follow-up Study:* E Rimm; *US Physicians' Health Study:* M Gaziano, J E Buring, C Hennekens; *Värmland:* S Törnberg, J Carstensen; *Whitehall:* M Shipley, D Leon, M Marmot; *Clinical Trial Service Unit (CTSU):* J Armitage, C Baigent, Z Chen, R Clarke, R Collins, J Emberson, J Halsey, M Landray, S Lewington, A Palmer (deceased), S Parish, R Peto, P Sherliker, G Whitlock.

Conflict of interest statement

All of the writing committee (except NQ) work in the CTSU, which has a policy of staff not accepting fees, honoraria, or consultancies. The CTSU is, however, involved in clinical trials of cholesterol modification therapy with funding from the MRC, BHF, and/or various companies (Merck, Schering, Solvay) as research grants to (and administered by) Oxford University. NQ works in Oxon Clinical Epidemiology Limited, and has stock options in Glaxo Smith Kline.

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The Presence of *BRAF* Point Mutation in Adult Papillary Thyroid Carcinomas From Atomic Bomb Survivors Correlates With Radiation Dose

Keiko Takahashi,[1,5] Hidetaka Eguchi,[1] Koji Arihiro,[6] Reiko Ito,[1] Kojiro Koyama,[2,8] Midori Soda,[3] John Cologne,[4] Yuzo Hayashi,[7] Yoshihiro Nakata,[5] Kei Nakachi,[1] and Kiyohiro Hamatani[1]*

[1]*Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, Hiroshima, Japan*
[2]*Department of Epidemiology (Hiroshima), Radiation Effects Research Foundation, Hiroshima, Japan*
[3]*Department of Epidemiology (Nagasaki), Radiation Effects Research Foundation, Nagasaki, Japan*
[4]*Department of Statistics, Radiation Effects Research Foundation, Hiroshima, Japan*
[5]*Department of Pharmacology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan*
[6]*Department of Pathology, Hiroshima University Medical Hospital, Hiroshima, Japan*
[7]*Department of Pathology, Hiroshima Asa Citizen's Hospital, Hiroshima, Japan*
[8]*Department of Urology, Oumu Municipal National Health Insurance Hospital, Hokkaido, Japan*

In papillary thyroid carcinogenesis, the constitutively activated mitogen-activated protein (MAP) kinase signaling pathway caused by a genetic alteration such as *RET/PTC* rearrangement or mutation of *RAS* and *BRAF* genes, is thought to be a major early event. Among these, the recently identified $BRAF^{V600E}$ mutation has been found at high frequency in adult patients with papillary thyroid carcinoma (PTC). However, the association between this mutation and radiation exposure in adult PTC is still unknown. In this study, we examined the $BRAF^{V600E}$ mutation in 64 PTCs among adult atomic bomb survivors in Hiroshima, Japan, comprising 17 nonexposed (0 mGy) and 47 exposed patients who developed the carcinoma after the bombing, and assessed the association of $BRAF^{V600E}$ mutation with clinicopathological and epidemiological variables. The median radiation dose in PTCs with the $BRAF^{V600E}$ mutation was significantly lower than that without the mutation (18.5 vs.156.9 mGy, Wilcoxon rank-sum test, $P=0.022$). A significant difference was found in the median latency period (years elapsed from atomic bombing to diagnosis) between exposed patients with and without $BRAF^{V600E}$ mutation (29 vs. 21 yr, Wilcoxon rank-sum test, $P=0.014$). These findings were further confirmed by logistic regression analysis with $BRAF^{V600E}$ mutation status as a dependent variable and taking into account possible interactions between the variables. We found that the log-transformed radiation dose and latency period were independently associated with the $BRAF^{V600E}$ mutation ($P=0.039$ and $P=0.010$, respectively). These results suggest that involvement of *BRAF* mutation in thyroid carcinogenesis in exposed people may differ from that in the nonexposed people.

Key words: $BRAF^{V600E}$ mutation; radiation dose; latency period; thyroid carcinogenesis

INTRODUCTION

Thyroid cancer is well-known to be associated with exposure to external or internal ionizing radiation, such as from the atomic bomb (A-bomb) or the Chernobyl accident. The excess relative risk of thyroid cancer per Sv was 1.15 in the Life Span Study of A-bomb survivors [1], and a strong relationship between thyroid cancer and radiation dose was indicated from the Chernobyl accident [2].

In papillary thyroid carcinogenesis, constitutive activation of the MAP kinase signaling pathway caused by a genetic alteration, including rearrangements of *RET/PTC* or mutation of *RAS* and *BRAF* genes, is thought to be a major early event [3–5]. Among these alterations, the *BRAF* gene mutation in the pathogenesis of papillary thyroid carcinoma (PTC) has recently gained considerable attention.

The *BRAF* gene encodes a serine/threonine kinase responsible for the transduction of signals in the MAP kinase cascade, which leads to the regulation of transcription factors, cytoskeletal elements, and other protein kinases that control cell proliferation [6]. *BRAF* somatic mutations were first discovered in several types of human cancers, including malignant melanomas, and colorectal and ovarian cancers [7].

Abbreviations: MAP kinase, mitogen-activated protein kinase; PTC, papillary thyroid carcinoma; A-bomb, atomic bomb; mGy, milli gray.

*Correspondence to: Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, 5-2, Hijiyama-Park, Minami-ku, Hiroshima, 732-0815, Japan.

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Except for very rare instances, the *BRAF* mutation identified in thyroid cancer so far is almost exclusively the thymine-to-adenine transversion at nucleotide 1799, resulting in the substitution of glutamate for valine at residue 600 (V600E) [8]. The V600E (formerly called V599E) substitution is thought to convert BRAF inactive conformation into its active form by disrupting the residue–residue interaction between the activation loop and the ATP binding site [9]. Recent data on the frequent prevalence of $BRAF^{V600E}$ mutation in thyroid microcarcinomas support the hypothesis that $BRAF^{V600E}$ mutation is an early event in PTC pathogenesis [10], along with the induction of goiter or invasive PTC in $BRAF^{V600E}$ transgenic mice [11].

$BRAF^{V600E}$ mutation has so far been described as occurring with a frequency ranging from 29 to 83% in PTC from adult patients [8]. In order to clarify the role of the $BRAF^{V600E}$ mutation in PTC, associations of the $BRAF^{V600E}$ mutation and clinico-pathological and epidemiological factors in PTC have been examined [8]. Among the common subtypes of PTC, prevalence of the $BRAF^{V600E}$ mutation showed a clear association with histological subtype. The highest frequency was in tall cell PTC (overall 77%), the second highest in conventional PTC (overall 60%), and the lowest in follicular variant PTC (overall 12%) [8], implying the possible role of $BRAF^{V600E}$ mutation in the determination of histology of PTC. The relationship between $BRAF^{V600E}$ mutation frequency and age at diagnosis in adult patients is still controversial. Both a significant correlation [12,13] and no correlation [14–17] have been reported. Thus, presence or absence of $BRAF^{V600E}$ mutation may be a key event for characterization of PTC, as is the case in colon cancer [18].

With regard to the relation to radiation exposure, the $BRAF^{V600E}$ gene mutation was studied in a type of radiation-related PTC, postChernobyl PTC, which is believed to have developed in those exposed to radiation as children. Very low frequencies of $BRAF^{V600E}$ mutation in this PTC have been reported (range: 0–12%) [19–23]. However, the prevalence of $BRAF^{V600E}$ mutation was also low (range: 0–6%) in PTC among children and adolescents who were not exposed to radiation [19,20,23]. A low prevalence of the $BRAF^{V600E}$ mutation has thus been observed in childhood PTC regardless of the presence or absence of past radiation exposure.

On the other hand, the association of the $BRAF^{V600E}$ mutation with radiation exposure has not been studied in adult PTC with history of radiation exposure. Because the prevalence of $BRAF^{V600E}$ mutation has been reported to be high in adult PTC [8], analysis of the association of $BRAF^{V600E}$ mutation with radiation exposure in adult PTCs is particularly important.

In this study, we compared clinico-pathological and epidemiological characteristics of adult PTC among A-bomb survivor patients by $BRAF^{V600E}$ mutation status.

MATERIALS AND METHODS

Tissue Specimens

Study subjects comprised 64 cases of adult PTC found among A-bomb survivors in Hiroshima. Classification of histology was done according to histopathological typing of the World Health Organization [24]. Study materials were formalin-fixed and paraffin-embedded thyroid tissue specimens obtained from the subjects between 2003 and 2005 under approval of the Human Investigation Committee and the Ethics Committee for Genome Research at the Radiation Effects Research Foundation (RERF).

DNA Preparation and Determination of $BRAF^{V600E}$ Mutation

Five-micrometer tissue sections were deparaffinized, stained with Methyl Green (Sigma-Aldrich, St. Louis, MO) and dissected manually or using laser microdissection system Leica AS LMD (Leica, Wetzler, Germany). DNA was extracted from the microdissected noncancerous or cancerous regions using QIAamp DNA Micro kit (QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) was performed in a 25 μL mixture containing 10 pmoles of each primer, 200 μM of each dNTP, 0.5 U of FastStart High Fidelity DNA polymerase (Roche, Basel, Switzerland), 20–50 ng of genomic DNA, and 1× reaction buffer supplied by the manufacturer. PCR conditions consisted of initial denaturation (95°C for 2 min), followed by 40 cycles (denaturation at 94°C for 30 s, annealing at 54°C for 60 s, extension at 72°C for 30 s). Primers used were 5′-tcatgaagacctcacagtaaaaat-3′ and 5′-tggatccagacaactgttcaa-3′. $BRAF^{V600E}$ mutation was initially screened by restriction fragment length polymorphism (RFLP) using restriction enzyme *Tsp*RI (New England Biolabs, Ipswich, MA) and was confirmed by direct sequencing using DNA sequencer CEQ8000 (Beckman Coulter, Inc., Fullerton, CA).

Statistical Analysis

Univariate analysis for comparison of clinico-pathological and epidemiological variables by radiation exposure or $BRAF^{V600E}$ mutation status was conducted using nonparametric tests (Wilcoxon rank-sum test) for continuous variables, because the distribution of radiation dose and latency period could not be assumed to be symmetrical. Fisher's exact was used for categorical variables. Logistic regression analysis was carried out among A-bomb survivor patients who were exposed to atomic

radiation, and we assessed the relationship between $BRAF^{V600E}$ mutation status and clinico-pathological and epidemiological variables including log- transformed radiation dose, latency period, histology, gender, and age at the time of A-bombing (or age at diagnosis). All statistical analyses were performed with SPSS software (version 12.0).

Radiation Dose

A-bomb radiation doses used in this analysis were estimated by the recently implemented DS02 system [25].

RESULTS

Noting that almost all PTC in A-bomb survivor patients occurred in adults, we examined the $BRAF^{V600E}$ mutation in 64 adult PTC cases among A-bomb survivor patients (cohort members of the Life Span Study) in Hiroshima, Japan, comprising 17 nonexposed (0 mGy) and 47 exposed patients (median dose: 150.7 mGy) who developed carcinoma after the bombing. All thyroid cancer tissue samples used were formalin-fixed, paraffin-embedded surgical specimens resected during 1956–1993. Patient characteristics such as gender, age at the time of A-bombing, age at diagnosis, latency period (years from A-bombing to diagnosis, being defined only for exposed patients), and histological subtypes are summarized in Table 1. All patients analyzed in this study were diagnosed at the age of 20 yr or older. All tumors were well-differentiated PTC including three cases of follicular variant; none was a solid variant.

DNA samples were extracted from microdissected specimens of cancerous or noncancerous tissue. We first conducted screening of $BRAF^{V600E}$ mutation by RFLP, followed by direct sequencing of the fragments to confirm the mutation (Figure 1). The frequency of $BRAF^{V600E}$ mutation in nonexposed patients (71%) was in good agreement with that reported for conventional adult PTC (Table 1) [8].

We then examined whether $BRAF^{V600E}$ mutation status in PTC in all A-bomb survivor patients consisting of both the nonexposed and the exposed people is related to any clinico-pathological or epidemiological characteristics, including radiation dose, age at the time of A-bombing, and age at diagnosis. The relationship between $BRAF^{V600E}$ mutation status and each factor is summarized in Table 2. The median radiation dose in PTC with $BRAF^{V600E}$ mutation was significantly lower than that in PTC without $BRAF^{V600E}$ mutation (Table 2, $P=0.022$). Furthermore, a marginally significant association was found between $BRAF^{V600E}$ mutation status and histological subtype (Table 2, $P=0.062$); all three cases of follicular variant harbored wild-type *BRAF*. This was in agreement with the previous observation of a low prevalence (12%) of $BRAF^{V600E}$ mutation in follicular variant PTC [8]. On the other hand, age at the time of A-bombing, age at diagnosis, and gender did not evidence significant association with $BRAF^{V600E}$ mutation status in the univariate analysis.

Additionally, an analysis on clinico-pathological or epidemiological characteristics including latency period that can be defined only for exposed patients was also undertaken in relation to $BRAF^{V600E}$ mutation with only the 47 exposed patients (Table 2). In addition to the characteristics found to be associated with $BRAF^{V600E}$ mutation status in all patients,

Table 1. Clinico-Pathological and Epidemiological Characteristics of Patients by Radiation Exposure Status

	Nonexposed patients[a] ($n=17$)	Exposed patients ($n=47$)	*P*-value
$BRAF^{V600E}$ mutation			
Present (*n*)	12	26	0.4[c]
Absent (*n*)	5	21	
Frequency (%)	71	55	
Median radiation dose (mGy, range)	0	150.7 (0.4–2758)	
Median latency period[b] (yr, range)	—	26.0 (11–46)	
Median age at the time of atomic-bombing (yr, range)	21.0 (5–52)	25.0 (1–49)	0.4[d]
Median age at diagnosis (yr, range)	48.0 (34–84)	54.0 (20–89)	0.6[d]
Histology			
Conventional PTC (*n*)	17	44	0.6[c]
Follicular variant (*n*)	0	3	
Gender			
Male (*n*)	1	5	1.0[c]
Female (*n*)	16	42	

[a]The nonexposed patients were either those with radiation dose estimated to be 0 mGy or those who were not in the city of Hiroshima at the time of bombing.
[b]Latency period: years from A-bombing to diagnosis.
[c]Fisher's exact test.
[d]Wilcoxon rank-sum test.

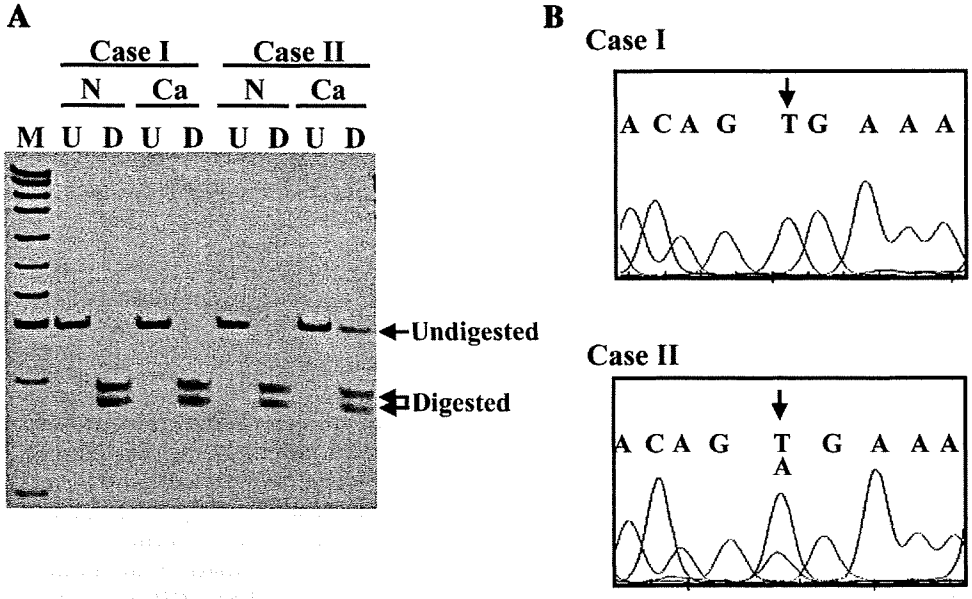

Figure 1. Detection of *BRAF*^{V600E} mutation. (A) RFLP analysis of *BRAF*^{V600E} mutation. DNA fragments containing nucleotide position 1799 were amplified and subsequently digested with restriction enzyme *Tsp*RI, as described in Materials and Methods. Representative results of gel electrophoresis of two samples are shown. N indicates noncancer; Ca, cancer; U and D, undigested and digested with *Tsp*RI; and M, molecular weight marker, respectively. Horizontal arrows indicate positions of undigested- or digested-bands. *Tsp*RI digests wild-type fragments, but not mutated ones. (B) Direct sequencing of PCR fragments. Sequences of the fragments amplified using DNA from tissue specimens of cases I and II are shown. Vertical arrow indicates nucleotide positions 1799. Heterozygous signal of T and A was detected for case II.

latency period also showed a statistically significant association with the mutation; the median latency period in PTC with *BRAF*^{V600E} mutation was significantly longer than that in PTC without the mutation (Table 2, $P=0.014$).

Because these clinico-pathological and epidemiological variables may be interrelated, we further performed multivariate logistic regression analysis for the 47 exposed patients, with *BRAF*^{V600E} mutation status as the dependent variable (Table 3). Log-transformed radiation dose was used as an explanatory variable, because the distribution of log-transformed radiation dose could be assumed to be nearly symmetrical, whereas that of nontransformed radiation dose could not. Log-transformed radiation dose showed a significant inverse association with *BRAF*^{V600E} mutation ($P=0.039$), and latency period revealed a significant positive association ($P=0.010$), confirming the results of univariate analyses shown above. The same result was obtained when age at the time of A-bombing was substituted for age at diagnosis as a variable in the regression analysis.

DISCUSSION

In papillary thyroid carcinogenesis, constitutive activation of the MAP kinase signaling pathway, namely *RET* and *NTRK* tyrosine kinase receptor rearrangements and *RAS* and *BRAF* oncogene activation, seems to be required for transformation [26]. Interestingly, mutual exclusion of these genetic events in the MAP kinase signaling pathway was reported between *BRAF* mutation and *RET/PTC* rearrangements, and between *BRAF* and *RAS* mutations [3–5,21,27–30]. Furthermore, a recently identified *AKAP9-BRAF* rearrangement was not shared with *BRAF* mutation in radiation-associated PTC [22]. Thus, no PTC case possessed more than one of the following mutational events: *BRAF*^{V600E}, *NTRK1* or *RET/PTC* rearrangements [5]. These data suggest that a single genetic event in the MAP kinase signaling pathway may be sufficient for thyroid cell transformation and tumorigenesis. Recent in vitro and in vivo experiments have also demonstrated the requirement of activation of the RET/PTC-RAS-BRAF-MAPK pathway in thyroid tumorigenesis [34–36].

In our study, we found that 71% (12/17) of PTC among nonexposed PTC patients had *BRAF*^{V600E} mutation, indicating that among the several events in the RET/PTC-RAS-BRAF-MAPK pathway, *BRAF*^{V600E} mutation is the most common for non-exposed adult Japanese PTC patients. On the other hand, *BRAF*^{V600E} mutation accounted for only 17% (2/12) of the adult PTC patients who were exposed to radiation dose greater than 500 mGy. These findings suggest that *BRAF*^{V600E} mutation is not a major event in the development of radiation-associated PTCs in adult patients, such as A-bomb survivors with high radiation exposure.

On the other hand, *RET/PTC* rearrangements have been shown to be particularly prevalent in PTCs from

Table 2. Clinico-Pathological and Epidemiological Characteristics of Patients by $BRAF^{V600E}$ Mutation Status

	All patients†			Exposed patients		
$BRAF^{V600E}$ mutation status	Present ($n=38$)	Absent ($n=26$)	*P*-value	Present ($n=26$)	Absent ($n=21$)	*P*-value
Median radiation dose (mGy, range)	18.5 (0–2,758)	156.9 (0–2,304)	0.022*	104.9 (0.4–2,758)	333.4 (0.7–2,304)	0.025*
Median age at the time of atomic-bombing (yr, range)	24 (1–52)	22.5 (3–49)	0.8*	29.5 (1–47)	24 (3–49)	0.9*
Median age at diagnosis (yr, range)	54 (20–89)	51 (29–70)	0.2*	54 (20–89)	51 (29–70)	0.2*
Latency period (yr, range)	—	—		29 (15–46)	21 (11–36)	0.014*
Histology						
Conventional PTC (*n*)	38	23	0.062‡	26	18	0.082‡
Follicular variant (*n*)	0	3		0	3	
Gender						
Male (*n*)	3	3	0.7‡	2	3	0.6‡
Female (*n*)	35	23		24	18	

†Patients consist of 17 nonexposed and 47 exposed patients.
*Wilcoxon rank-sum test.
‡Fisher's exact test.

Table 3. Logistic Regression Analysis of $BRAF^{V600E}$ Mutation Status*

	β[a]	*P*-value
Radiation dose (log_{10} transformed)	−0.979	0.039
Latency period	0.124	0.010
Age at the time of atomic-bombing	0.031	0.4
Gender	−0.685	0.7
Histology	−23.948	1.0

*Analysis was performed only for the 47 exposed patients.
[a]Regression coefficients in the logistic regression model.

post-Chenobyl children and from patients with a history of radiation therapy [31–33]. In addition, *AKAP9-BRAF* rearrangement was recently found in PTCs in post-Chernobyl children [22]. Moreover, in vitro and in vivo experiments have revealed that external X-ray irradiation can induce rearrangement of *RET/PTC1* and *RET/PTC3* in tumor cell lines and human normal thyroid tissue transplanted in scid mice [37–39]. These findings suggest that chromosomal rearrangements may be important in the development of radiation-associated papillary thyroid cancer. Thus, we hypothesize that radiation exposure may influence the selection of an early genetic event; a genetic event other than $BRAF^{V600E}$ mutation in the MAP kinase signaling pathway including chromosomal rearrangements may be involved in the development of PTCs among A-bomb survivors, specifically those exposed to high radiation dose.

We found a significant association of $BRAF^{V600E}$ mutation with latency period (years from A-bombing to diagnosis) among exposed patients (Table 2). Notably, latency period was positively associated with $BRAF^{V600E}$ mutation in logistic regression analysis including age at the time of A-bombing or age at diagnosis as a covariable (Table 3). The low frequency of $BRAF^{V600E}$ mutation in PTCs with short latency period also suggests that a molecular event other than $BRAF^{V600E}$mutation in the MAP kinase signaling pathway may play a major role in the development of PTC among A-bomb survivors.

This study has several limitations: PTC specimens resected before 1956 are unavailable, rendering it impossible to assess PTC developed within 10 yr after the A-bombing. Other molecular events, specifically *RET/PTC* rearrangement and *RAS* mutations, need to be analyzed with an increased number of study subjects. Our findings in this study therefore argue for the need to do further studies to clarify the mechanisms of radiation-associated PTC.

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Masatsugu Yano[a,*], *Kiyohiro Hamatani*[b], *Hidetaka Eguchi*[b], *Yuko Hirai*[b], *Donald G. MacPhee*[b], *Keizo Sugino*[a], *Kiyohiko Dohi*[a], *Toshiyuki Itamoto*[c], *Toshimasa Asahara*[c]

[a]*Department of Surgery, Akane Foundation Tsuchiya General Hospital, 3-30 Nakajima-chou, Naka-ku, Hiroshima 730-8655, Japan*
[b]*Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation (RERF), Hiroshima, Japan*
[c]*Department of Surgery, Division of Frontier Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*

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[a]*Department of Surgery, Akane Foundation Tsuchiya General Hospital, 3-30 Nakajima-chou, Naka-ku, Hiroshima 730-8655, Japan*
[b]*Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation (RERF), Hiroshima, Japan*
[c]*Department of Surgery, Division of Frontier Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*

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ABSTRACT

Association of gene alterations and prognosis has not fully been elucidated in hepatocellular carcinoma (HCC). To clarify the relationship between *p53* and *hMSH2* mutations and prognosis, we analysed these mutations in 83 HCC cases and assessed their association with various clinicopathological factors. The 3-year disease-free survival (DFS) or overall survival (OS) rates in HCC patients with *p53* mutation and *p53* wild/*hMSH2* mutation significantly decreased compared with those without these mutations (14.3% and 37.5% versus 67.5% for DFS; 35.7% and 50.0% versus 96.4% for OS, respectively). In the multivariate analysis, categories by *p53* and *hMSH2* mutation status, and liver cirrhosis demonstrated statistical significances for DFS and OS. Moreover, the frequency of patients with *p53* and/or *hMSH2* mutations in intrahepatic metastasis (75.0%) was significantly higher than that in multicentric occurrence (14.3%). Thus, *p53* and *hMSH2* mutations will be useful for identifying subsets of HCC patients with poor prognosis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, especially in Asia and Africa. Hepatocarcinogenesis seems to be a multi-step process where normal hepatocyte is transformed through hepatitis, cirrhosis and adenomatous hyperplasia into malignant tumour and then clinical liver cancer.[1] The various risk factors associated with the development of HCC are well known. They mainly include chronic HCV and HBV infection, heavy alcohol intake, prolonged exposure to aflatoxin B1 (AFB1) and metabolic liver diseases such as hemochromatosis. HCC development is closely associated with cirrhosis, and 80–90% of HCC are found in a chronic hepatitis or a cirrhotic liver.

The HCC, as well as precursor benign lesions, have been extensively studied in terms of genetic alteration in the past 10 years. As in other solid tumours, genetic abnormalities including genomic instability, gene alterations and aberrant expression of genes are accumulated during the carcinogenesis process. Indeed, chromosomal aberrations with loss of heterozygosity have been found in many cirrhotic livers and dysplastic nodules as well as HCC.[1–3] Furthermore, genetic alterations including *p53* family, *Wnt* pathways and DNA mismatch repair genes have been detected in the cirrhotic and dyplastic nodules, and HCC.[1,4–7]

P53 behaves as a multifunctional transcription factor involved in the control of cell cycle, programmed cell death, senescence, differentiation, DNA replication, DNA repair

* *Corresponding author:* Tel.: +81 82 243 9191; fax: +81 82 241 1865.
E-mail address: yano3masa@ybb.ne.jp (M. Yano).
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and maintenance of genomic stability. P53 mutation is shown to be associated with the progression of HCC from an early to a more advanced stage.[1] In HCC developed from populations exposed to AFB1, specific TP53 mutation R249S is observed in more than 50% of the tumours.[1] Microsatellite instability (MSI) occurs in hepatocytes in some cases of chronic hepatitis, cirrhosis, and HCC.[8,9] Alterations in DNA mismatch repair genes involved in MSI have also been found in HCC, especially HCV-associated HCC.[10] In this way, genetic alterations involved in some of the multi-steps in carcinogenesis have been elucidated to some extent in recent years. Understanding of the molecular mechanisms of HCC development is very important for the improvement in prevention, treatment strategies and prognosis of HCC.

There have been great recent advances made in treatment of HCC, but the long-term prognosis after curative resection of HCC remains poor. One of the primary reasons for poor prognosis following curative resection is the high recurrence rate—roughly 20–30% within 1 year and 80% in 5 years.[11,12] Intrahepatic recurrences arise from either intrahepatic metastases or multicentric occurrences. Recurrences due to intrahepatic metastases are generally found to be more aggressive than those from multicentric occurrences possibly because intrahepatic metastases are in a later stage of hepatocarcinogenesis than those of multicentric occurrences, which can be considered *de novo* tumours.[13,14] Distinction of these two types of recurrence is important not only for understanding the biological process of liver carcinogenesis, but also for determining the optimal treatment for the patient.

Our previous study showed that most mutations of not only *p53* but also *hMSH2* genes occurred in moderately or poorly differentiated HCCs, suggesting that the presence of either a *p53* or *hMSH2* gene mutation is involved in the tumour progression of HCC.[4] It was also suggested that lack of mutations in both *p53* and *hMSH2* closely correlates with the survival in HCC patients treated by surgery.[4] Among DNA mismatch repair genes, there seems close interaction between p53 and hMSH2 proteins during carcinogenesis. First, p53 alterations are associated with altered expression of MMR protein, namely hMSH2 protein.[7,15] Second, MSI inversely correlates with the presence of *p53* mutation in tumours.[16] Third, the presence of p53 response element in the *hMSH2* proximal promoter suggests that p53 regulates hMSH2 expression.[17,18] Fourth, p53 overexpression was associated with upregulation of hMSH2 protein.[7,15] Therefore, we hypothesise that alterations of *p53* and *hMSH2* genes may be associated with not only survival in HCC patients but also recurrence of HCC following curative resection.

The purpose of this study is to examine the effects of deficiencies of *p53* and *hMSH2* gene function on the development of recurrences of HCC and overall survival following curative resection and establish a relatively simple predictive assay which may be of value in the clinical care of HCC patients.

2. Materials and methods

2.1. Subjects

We obtained tissue samples by surgical resection from 83 HCC patients, in all cases with their informed consent. Histopathologic examination of haematoxylin-eosin stained, paraffin-embedded sections was performed for all patients. Tumours were histologically classified into well, moderately, and poorly differentiated HCC according to the criteria of Edmondson and Steiner.[19] Histological grading is classified by acidophilic cytoplasm, nuclear/cytoplasm ratio and arrangement of neoplastic cell. No patient was lost to follow-up. The duration of follow-up period is 36–105 months. The patient group consisted of 67 men and 16 women (ages 48–77 years; mean 63.0 ± 7.0). Serological testing for serum hepatitis B virus surface antigen was positive in 13 patients (15.7%), and serum anti-hepatitis C virus antibody was present in 64 patients (77.1%); we also found that two patients (2.4%) were positive for both markers and four patients (4.8%) were negative for both. Fifty of our patients (60.2%) had cirrhosis. Times to relapse and survival were measured from the date of surgery. For survival analyses, one patient who died of disease complication within a month of surgery and three patients whose resections were non-curative were excluded.

2.2. DNA isolation

Samples of dissected tumours and surrounding non-cancerous tissues were frozen in liquid nitrogen and stored at −80 °C until their DNA was extracted. Genomic DNA was digested with SDS and proteinase K prior to extraction with phenol–chloroform and precipitation with ethanol. After extraction, the purified DNA was stored at 4 °C.

2.3. Single-strand conformational polymorphism (SSCP)

To screen the *hMSH2* and *p53* genes for variant sequences, we performed SSCP analysis by the method of Orita and colleagues[20], with particular emphasis on all coding exons of the *hMSH2* gene as well as exons 5–8 of the *p53* gene. The PC primers for amplification of each exon of *hMSH2* and *p53*, and the polymerase chain reaction (PCR) conditions were as previously described.[21,22] PCR-amplified fragments were heat denatured at 95 °C for 10 min and then loaded on to 8% non-denaturing polyacrylamide gels maintained at 5 °C; the gels were dried and exposed to X-ray film. Samples exhibiting altered SSCP migration patterns were subjected to direct nucleotide sequencing.

2.4. Direct nucleotide sequencing

After purification of the PCR products, the products were used as templates for sequencing. For *p53*, the PCR products were denatured to produce single-stranded templates before fluorescence sequencing was performed in an automated sequencing system (ALFred DNA Sequencer, Pharmacia LKB, Uppsala, Sweden). The dideoxy chain-termination method and the Thermo sequenase fluorescent labelled primer cycle sequencing kit were used (Amersham Life Science, Little Chalfont, England).

2.5. Statistics

The Chi-square or Fisher's exact test was used to evaluate the statistical significance of categorical variables. Cumulative

disease-free and survival rates were estimated by the method of Kaplan and Meier. The statistical significance of differences in the survival curves of different subgroups was analysed by the log-rank test. The overall survival of the study variables was assessed using the Cox proportional hazards model. Multiple group comparisons were conducted by one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test. These statistic analyses were performed with SPSS ver. 11.5J software.

3. Results

3.1. *Mutations of the p53 and hMSH2 genes in HCC*

We screened the genomic DNA from 83 HCC patients for somatic mutations in the *p53* and *hMSH2* genes by both PCR–SSCP and direct sequencing using primer sets shown in Table 1. Tables 2 and 3 summarise results of these mutations. We detected mutations of the *p53* gene in 16/83 patients (19.3%); 12 of the 16 mutations were missense, three were deletions and one was nonsense (Table 2). Among these *p53* mutations, we found triple missense mutations in one case (#57) and double ones in another (#69) (Table 2). On the other hand, point mutations of the *hMSH2* gene were found in 9/83 patients (10.8%) (Table 3). Only one patient had both *p53* and *hMSH2* gene mutations in his tumour.

3.2. *Clinico-pathological characteristics by mutation status of p53 and hMSH2 genes*

To assess the association of mutation status of *p53* and *hMSH2* genes with clinico-pathological characteristics, HCC patients were divided into three groups; namely, those with *p53* mutation ($n = 16$) including one case possessing both *p53* and *hMSH2* mutations, with *p53* wild/*hMSH2* mutation ($n = 8$), and with *p53* wild/*hMSH2* wild ($n = 59$). Presence or absence of intrahepatic metastasis (im) showed a significant heterogeneity in the distribution of number of patients among three groups ($P = 0.026$, chi-square test) (Table 4). In addition, when compared HCC patients harbouring *p53* and/or *hMSH2* mutations and those without mutations, there was a significant difference only for im ($P = 0.015$, Fisher's exact test) (Table 4).

Table 2 – Mutations of *p53* gtene in HCC

Case No.	Exon	Codon	Nucleotide alterations	Amino acid substitutions
4	6	220	TAT → TGT	Tyr → Cys
6	7	237	ATG → ATT	Met → Ile
25	5	176	TGC → TAC	Cys → Tyr
31	5	164	AAG → TAG	Lys → Stop
32	7	246	ATG → GTG	Met → Val
35	8	281	GAC → TAC	Asp → Tyr
39	6	189	1 bp deletion (frame shift)	
42	5	146	TGG → TGT	Trp → Cys
44	8	283	1 bp deletion (frame shift)	
52	5	155	ACC → ATC	Thr → Ile
56	6	214	CAT → CGT	His → Arg
57	6	200	AAT → AGT	Asn → Ser
	6	205	TAT → TCT	Tyr → Ser
	6	207	GAT → GCT	Asp → Ala
68	7	238	TGT → CGT	Cys → Arg
69	5	173	GTG → ATG	Val → Met
	5	175	CGC → ATG	Arg → Cys
72	5	154–157	9 bp deletion	
76	5	155	ACC → ATC	Thr → Ile

Table 1 – Oligonucleotide primer sequences used for detection of alterations of *p53* and *hMSH2* genes

p53	Sense (5′ → 3′)	Antisense (5′ → 3′)	Length (bp)
Exon 5	TCCTACAGTACTCCCCTGCC	GGCCCAGCTGCTCACGATC	207
Exon 6	ACTGATTGCTCTTAGGTCTG	AGTTGCAAACCAGACCTCAG	143
Exon 7	AGGTTGGCTCTGACTGTACC	GTCCTGACCTGGAGTCTTCC	120
Exon 8	CTATCCTGAGTAGTGGTAATC	GTCCTGCTTGCTTACCTCGC	165
hMSH2	Sense (5′ → 3′)	Antisense (5′ → 3′) Length	(bp)
Exon 1	TCGCGCATTTTCTTCAACCA	TCCCTCCCCAGCACG	284
Exon 2	TTTTTTGAGCAAAGAATCTGG	ACCTTATATGCCAAATACCAATC	162
Exon 3	TTAGGCTTCTCCTGGCAATC	CCTTTCCTAGGCCTGGAATC	332
Exon 4	CTTATTCCTTTTCTCATAGTAGT	TTGTAATTCACATTTATAATCCATG	221
Exon 5	GCTATAGGAAATCTTCGATTTTTA	TACCTAAAAAGGTTAAGGGCTC	193
Exon 6	TGAGCTTGCCATTCTTTCTATT	TGGGTAACTGCAGTTACATAAA	225
Exon 7	TTCAGATTGAATTTAGTGGAAGC	ACCTTCATGTTTTTCCAGAGC	207
Exon 8	TTTGTTTTACTACTTTCTTTTAGG	AAGTATATTGCATACCTGATCC	148
Exon 9	TAATTTCTGTCTTTACCCATTATTT	CAACCTCCAATGACCCATTC	204
Exon 10	TGGTAGTAGGTATTTATGGAATAC	ATCATGTTTAAGAGCATTTAGGG	264
Exon 1 1	TACACATTGCTTCTAGTACAC	AGCCAGGTGACATTCAGAAC	202
Exon 12	ATTATTCAGTATTCCTGTGTAC	ACCCCCACAAAAGCCCAAA	326
Exon 13	ATTTATTAGTAGCAGAAAGAAGTT	AAGGGACTAGGAGATGCAC	287
Exon 14	GTTACCACATTTTATGTGATGG	TTCTGAATTTAGAGTACTCC	329
Exon 15	TCTCATGCTGTCCCCTCAC	AAGTTAAACTATGAAAACAAACTG	247
Exon 16	ACTAATGGGACATTCACATGTG	TCAATATTACCTTCATTCCATTAC	232

Table 3 – Mutations of MSH2 gene in HCC

Case No.	Exon	Codon	Nucleotide alterations	Amino acid substitutions
1	1	45	GCG → GTG	Ala → Val
14	7	390	CTT → TTT	Leu → Phe
22	7	390	CTT → TTT	Leu → Phe
	12	629	CAA → CGA	Gln → Arg
33	14	803	ACA → GCA	Thr → Ala
39	3	180	CCA → ACA	Pro → Thr
59	3	180	CCA → TCA	Pro → Ser
64	3	191	CAT → CGT	His → Arg
73	3	180	CCA → ACA	Pro → Thr
81	3	180	CCA → ACA	Pro → Thr

Except for the im, none of the other variables showed any difference among these groups.

3.3. *Disease-free survival*

We conducted univariate analysis for disease-free survival (DFS) in all 79 HCC patients with follow-up data available (Table 5). Presence or absence of *p53* mutation showed a significant difference of DFS period (median: 5 versus 48 months, $P < 0.0001$, log-rank test). On the other hand, other variables including *hMSH2* mutation did not show any significant differences for DFS. Since *p53* mutation status was such a strong prognostic factor, stratification of HCC patients by *p53* mutation status is required not to overlook other prognostic factors. We then analysed only for 65 HCC patients without *p53* mutation. Notably, HCC patients with *hMSH2* mutation showed a significantly shorter DFS than those without the mutation (18 versus 58 months, $P = 0.019$). In addition, liver cirrhosis showed a marginal significance among 65 HCC patients with wild *p53* ($P = 0.075$, log-rank test).

Comparisons of DFS among three groups of HCC patients with *p53* mutation ($n = 14$), those with *p53* wild/*hMSH2* mutation ($n = 8$) and those without mutation ($n = 57$) are shown using Kaplan–Meier survival curves (Fig. 1). The 3-year DFS rates of HCC patients were 14.3%, 37.5% and 67.5%, respectively.

In the multivariate analysis, categories by *p53* and *hMSH2* mutation status and liver cirrhosis that showed $P < 0.1$ in univariate analysis were included as explanatory variables (Table 6). HCC patients with *p53* wild/*hMSH2* mutation and those with *p53* mutation showed 2.9- and 7.3-fold risks as compared with those without mutation ($P = 0.014$, $P < 0.001$, respectively). In addition, liver cirrhosis also showed a statistically significant difference ($P = 0.025$) in the analysis.

3.4. *Association of recurrent patterns and mutation status of p53 and hMSH2 genes*

Recurrence was detectable in 45 of 79 patients (57.0%). As shown in Table 7, frequency of recurrence in *p53* and/or *hMSH2* mutation group was significantly higher than that in the *p53* wild/*hMSH2* wild group (87.0% versus 44.6%, $P = 0.001$). Moreover, interestingly, the frequency of patients

Table 4 – Clinico-pathological and epidemiological features stratified by *p53* and *hMSH2* mutation status

		p53 wild/ *hMSH2* wild (Reference) ($n = 59$)	*p53* mutation ($n = 16$)	*p53* wild/ *hMSH2* mutation $n = 8$	p^*	*p53* and/or *hMSH2* mutation ($n = 24$)	p^{**}
Gender	Female (*n*)	11	3	2	0.9	5	1
	Male (*n*)	48	13	6		19	
Age	⩽69 yrs (*n*)	45	15	5	0.2	20	0.6
	⩾70 yrs (*n*)	14	1	3		4	
Tumour size	<2 cm (*n*)	10	0	1	0.2	1	0.2
	>2 cm (*n*)	49	16	7		23	
Differentiation	Well (*n*)	11	0	1	0.2	1	0.2
	Moderately/Poorly (*n*)	48	16	7		23	
HBs Ab	Negative (*n*)	48	14	7	0.8	21	0.7
	Positive (*n*)	11	2	1		3	
HCV	Negative (*n*)	15	3	1	0.6	4	0.6
	Positive (*n*)	44	13	7		20	
Liver cirrhosis	Negative (*n*)	20	9	4	0.2	13	0.1
	Positive (*n*)	39	7	4		11	
Intrahepatic metastasis	Negative (*n*)	36	4	3	0.026	7	0.015
	Positive (*n*)	23	12	5		17	
Portal vein involvement (*n*)	Negative (*n*)	39	7	5	0.3	12	0.2
	Positive (*n*)	20	9	3		12	

im, intrahepatic metastasis; vp, portal vein invasion.
* Chi-square test for 2 × 3 table.
** Fischer's exact test for 2 × 2 table (p53 wild/hMSH2 wild versus p53 and/or hMSH2 mutation).

Table 5 – Prognostic factors of disease-free and overall survival by uni variate analysis

Variables		All ($n = 79$)					Only p53 wild ($n = 65$)				
		No. of Patients	DFS		OS		No. of Patients	DFS		OS	
			Median (months)	P*	Median (months)	P*		Median (months)	P*	Median (months)	P*
Gender	Female (n)	16	36	0.9	67	0.5	13	41	0.9	98	0.9
	Male (n)	63	44		–		52	48		–	
Age	⩽69 yrs (n)	61	40	0.4	78	0.8	48	47	0.8		0.9
	⩾70 yrs (n)	18	48		98		17	48		98	
Tumour size	<2cm (n)	10	44	0.2	–	0.3	10	44	0.5	–	0.6
	⩾2cm (n)	69	38		78		55	48		98	
Differentiation	Well (n)	12	44	0.5	67	0.7	12	44	0.9	67	0.7
	Moderately/Poorly (n)	67	41		98		53	58		98	
HBs Ag	Negative (n)	65	40	0.9	78	0.3	53	48	0.9	98	0.4
	Positive (n)	14	44		–		12	47		–	
HCV	Negative (n)	18	58	0.2	–	0.068	16	58	0.2	–	0.1
	Positive (n)	61	32		67		49	44		98	
Liver cirrhosis	Negative (n)	33	47	0.3	–	0.037	24	–	0.075	–	0.008
	Positive (n)	46	32		61		41	41		67	
im	Negative (n)	42	44	0.5	98	0.7	38	47	1.0	98	1.0
	Positive (n)	37	36		78		27	48		–	
vp	Negative (n)	49	44	0.7	–	0.1	43	47	0.6	–	0.4
	Positive (n)	30	20		78		22	–		98	
p53	Wild (n)	65	48	<0.0001	98	<0.0001					
	Mutated (n)	14	5		24						
hMSH2	Wild (n)	70	44	0.3	98	0.3	57	58	0.019	98	0.011
	Mutated (n)	9	21		–		8	18		30	

DFS, disease-free survival; OS, overall survival; im, intrahepatic metastasis; vp, portal vein invasion.
A dash (-) indicates that the median survival could not be calculated because the last cumulative survival was greater than 50%.
* Log-rank test.