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Solid Cancer Incidence among Atomic Bomb Survivors: Preliminary Data from a Second Follow-Up

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More than half a century after the atomic bombings in Hiroshima and Nagasaki, an increased risk of cancer incidence is still apparent among the Life Span Study (LSS) cohort of survivors. Although a great deal has been learned from the long follow-up of the LSS cohort, questions regarding radiation-related cancer risks still remain. We are conducting a second comprehensive cancer incidence follow-up to help answer some of these questions. Since the 1987 follow-up, there was a 24% increase in person-years and 56% increase in cancer cases. With the additional 11 years of follow-up, i.e. now including the years from 1958 to 1998, almost 17,500 first primary solid cancers were identified among over 105,000 LSS members with estimated DS02 organ doses.

The LSS cohort includes 120,321 people including about 50,000 survivors who were within 2.5 km of the bombings, about 45,000 who were within 2.5-10 km, and also about 25,000 who were not in either Hiroshima or Nagasaki at the time of the bombings, the so-called Not-In-City (NIC) group. In the past, the NIC group was not included in most of the overall comprehensive studies, but they are included in the second follow-up because they can improve inference about baseline risk patterns.

There are several important strengths of the LSS cohort. It is a large, healthy non-selected population that includes all ages and both sexes (though there are more females due to the fact that many male soldiers were not in the cities of Hiroshima and Nagasaki); members were exposed to a wide range of doses and they have well characterized dose estimates; mortality follow-up is virtually complete since 1950; cancer incidence ascertainment is complete in Hiroshima and Nagasaki tumor registry catchment areas since the establishment of the registries in 1958, and there is more than 50 years of follow-up.

When studying cancer incidence or mortality, certain differences in methods should be noted. For evaluating cancer incidence, we must exclude people who either died or had cancer diagnosed before the cancer registries were established in 1958. Therefore, there are about 8,000 fewer people in incidence analyses than in mortality analyses. Also, the mean age at the time of the bombing is a little younger in the survivors included in the incidence (26.8 years) compared with mortality (29.0 years) analyses because people who developed cancer before 1958 tended to be old and, as already mentioned, they are excluded from the incidence analyses.

Cancer incidence ascertainment is based on the LSS Tumor Registry. This registry includes all cancer cases diagnosed among LSS members registered in either the Hiroshima or Nagasaki Tumor Registries. The Hiroshima and Nagasaki Tumor Registries are of high quality because they employ active case identification in all large hospitals in their catchment areas. Data from tissue registries, death certificates, and medical associations (for the small hospitals) are also collected. Earlier analyses demonstrated that there is no dose bias in case ascertainment. Mortality data are obtained from the family registry (called Koseki) and they are nationwide.

The LSS cancer incidence studies add a valuable component to radiation risk assessment of the atomic bomb survivors because they include data on non-fatal cancers, some of which are quite radiation sensitive. Cancers of the breast, thyroid and skin, for example, are radiation sensitive but since they have very good survival a large number of them would be missed if only mortality data were evaluated. The incidence data are characterized by a high level cancer ascertainment, accurate diagnoses, information on histology, and long follow-up. For some organs, information on benign tumors also is collected.

The LSS cancer incidence studies do have some limitations. In particular, solid cancer data from 1945 to 1958 and leukemia data from 1945 to 1950 are incomplete, cancer ascertainment is limited to Hiroshima and Nagasaki area residents, and treatment data are limited. This means that some early cancer cases have been missed, especially leukemia and thyroid cancers which have a short latency period.

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The second comprehensive cancer incidence report includes follow-up from 1958 to 1998, with data on 105,427 people; 50% of whom were still alive in 1998 (currently about 45% are alive). Of note is that about 85% of individuals less than 20 years of age at the time of bombing were still alive in 1998 (about 80% today). In this report, we are studying only first primary tumors to prevent confounding from radiation treatment for the first cancer and possible detection bias in persons who already have cancers. All analyses in this report are based on the new DS02 dosimetry system which has incorporated several important improvements over DS86. Improvements in DS02 include refinements in the shielding calculations, transport calculations, and source term adjustment. In DS02, gamma doses increased and neutron doses decreased slightly. We used weighted colon dose in Gy to evaluate solid cancer and weighted organ doses for most site-specific analyses.

Table 1 shows the study population by dose categories. Excluding the non-exposed NIC group, 35,545 (slightly over 44% of the 80,180 exposed LSS members) A-bomb survivors were exposed to less than 0.005 Gy and 63,334, or 79% of the exposed cohort, were exposed to less than 0.1 Gy. Thus, the LSS is not such a high dose study as some may think, and it can provide substantial information on low dose radiation.

We used Poisson regression analysis to estimate the excess relative and absolute risks of all solid cancers combined and of individual cancer sites. The excess relative risk (ERR) quantifies the percentage change in risk for a unit of dose, in this case in Gy, i.e. it shows the relative change in cancer rates. The excess absolute rate (EAR) quantifies the absolute change in rates for a unit of dose, i.e. it shows the difference in cancer rates. The ERR and EAR can vary with age at exposure, gender, attained age, and other factors. They are both important and provide complementary information. In the analyses, we adjusted the person years of follow-up for the estimated migration of persons out of the Hiroshima and Nagasaki areas. We used a linear dose-response model as our standard, and considered the modifying effects of gender, attained age, age at exposure, and time since exposure.

In the second follow-up, 17,448 cancers were identified among the LSS cohort members (Table 2). The largest group of tumors (n=10,052) is of the digestive system, and stomach cancer which is a very common cancer in Japan was the most frequent cancer of the digestive tract. There were over 1000 cancer cases of the respiratory system, female genital organs, and breast cancer.

For all solid cancers combined, the dose response was linear and we saw no evidence of non-linearity. A statistically significant dose response trend was seen in the 0 - 0.15 Gy range, and this trend was consistent with that observed for the full dose range. The ERR per weighted colon dose in gray (ERR/Gy) for solid cancer was higher for women than men and decreased with increasing age at exposure and attained age. The EAR per 10,000 person years per weighted colon dose in Gy (EAR/10^4 PY Gy) was also higher among women and decreased with increasing age at exposure, but increased with increasing attained age. When gender-specific cancers were excluded from the analyses, the ERR/Gy remained significantly higher for

Table 1. Dose distribution in the LSS incidence cohort

Dose (Gy)	Number of Subjects	Percentage (%)
Not in city	25,247	23.9
< 0.005	35,545	33.7
0.005 - 0.1	27,789	26.4
0.1 - 0.2	5,527	5.2
0.2 - 0.5	5,935	5.6
0.5 - 1	3,173	3.0
1 - 2	1,647	1.6
2+	564	0.5
Total	105,427	100

Table 2. Distribution of solid cancers identified among the LSS cohort members during the period of 1958-1998

Site	Number of subjects
Digestive system	10,052
Respiratory system	2,001
Female genital	1,457
Breast	1,082
Urinary system	741
Thyroid	471
Skin	347
Male genital	420
Oral cavity	277
Nervous system	281
Other solid cancers	319
Total	17,448

females than males, but the gender difference disappeared when an absolute risk model was used. Lifetime solid cancer risk estimates appear to be about 20 times higher than those observed for leukemia.

As a result of the second follow-up, there is now a suggestion of an excess relative risk for endometrial cancer among women exposed before age 20. We also have identified radiation effects for male breast cancer, and found strong evidence that some time patterns differ when using the ERR and the EAR models. Using an EAR model, risk increased with increasing age, whereas the risk decreased with an ERR model.

Patterns of organ (or site) specific risks generally were similar to those seen in the previous follow-up, but the risk patterns have become clearer for some cancers. High ERRs were found for cancers of the bladder, breast and lung, while high EARs were seen for cancers of the stomach, breast, colon and lung. Assessing site-specific cancer risks is important, but because there are considerably fewer cases, it is difficult to identify significant differences in risk estimates or patterns. Biologically it is almost certain that variation in site-

specific risks exists, while current analyses suggest some differences much of the observed variability is consistent with random variation because formal statistical tests generally lack the power to detect real differences.

In summary, the updated solid cancer incidence data indicate that the shape of the dose response is well described by a linear model. Solid cancer excess rates increased throughout life for all ages, while excess relative risks decreased with increasing age. Excess risks for all solid cancers were higher for women than men, and lifetime risk estimates were considerably larger than for leukemia. The relatively small number of cancers for most individual sites made it difficult to identify statistically significant differences in age-time patterns. While overall patterns were similar to those seen in previous analyses, we continue to find new results with each new follow-up.

A large proportion of the radiation-associated excess solid cancers are likely to occur over the next 15 to 20 years. We therefore expect that the accumulating data will continue to offer important new insights into radiation effects on cancer risks. Continued follow-up is necessary to understand risk patterns for persons less than age 20 years at the time of the bombings. Additional site-specific incidence studies incorporating pathological reviews will provide needed information on the radiation-sensitivity of specific histologies. With close collaboration among statisticians, epidemiologists, biologists and pathologists; we should be able to improve our understanding of these data and their implications for radiation protection.

RESEARCH COMMUNICATION

Minimal Sizes of Cases with a Susceptible Genotype and Minimal Odds Ratios among Susceptible Individuals in Case-control Studies

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Abstract

Objective: Disease risk elevation due to an environmental factor only for individuals with a susceptible genotype is a typical example of gene-environment interaction. In order to identify risk factors interacting with susceptible genotypes in case-control studies, presumptions on minimal size of cases with the susceptible genotype (S_{min}) and odds ratio (OR) among the susceptible individuals ($OR_{susceptible}$) are useful.

Model: Proportion of exposed cases (P_1) and OR for whole cases (OR_{whole}) statistically detectable in a case-control study can be calculated in a conventional method. P_1 was assumed to be a weighted sum of the exposed among cases with the genotype (P_x) and cases without the genotype (equal to proportion of the exposed among controls, P_0), i.e., $S P_x + (1 - S) P_0$, where S is the size (proportion) of cases with the genotype. For each calculated P_1, S became the minimum (S_{min}) in case of $P_x = 1$. $OR_{susceptible}$ was calculated by $\{P_x (1 - P_0)\} / \{(1 - P_x) P_0\}$.

Results: S_{min} and $OR_{susceptible}$ were listed for the combinations of the above components. For example, a detectable P_1 was 0.638 for P_0=0.5 in a case-control study with 200 cases (N_1) and 200 controls (N_0), when α error of a two-sided test was 0.05 with an 80% of power. In case of P_1=0.638, OR_{whole} was 1.77, producing S_{min}=0.277 for infinite $OR_{susceptible}$. It indicates that an environmental factor cannot be detected in case that a high-risk genotype frequency is less than 0.277.

Interpretation: If the size of cases with a susceptible genotype is expected to be less than S_{min}, case-control studies are unlikely to detect a significant OR of the environmental factor.

Key Words: gene-environment interaction – genetic polymorphism – sample size – case-control studies

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Introduction

Recent development of genotyping methods allows us to examine the hypothesis that environmental factors cause a disease for individuals with a susceptible genotype. Although not perfect, it was exemplified by the finding that smoking causes lung cancer more frequently in those with low enzyme activity genotypes of carcinogen detoxification enzyme genes (Kiyohara et al., 2002; Mohr et al., 2003). Epidemiologically, such phenomena are termed as a gene-environment interaction, which is defined with a relative risk ratio of environmental exposure for those with a genotype relative to those without it, or a relative risk ratio of genotype for the exposed relative to the unexposed (Khoury and Flanders, 1996; Hamajima et al., 1999; Brennan, 2002). Since the elucidation of the interactions is useful for individualized disease prevention, researches on the interactions have been becoming popular in the field of epidemiology (Mucci et al., 2001; Kang, 2003). The targeted genotypes are selected from commonly observable ones, which are called "polymorphism" genotypes.

When the genotype interacting with an environmental factor is known, a sample size to detect the odds ratio (OR) of the factor in a case-control study can be calculated based

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on the genotype frequency with a conventional method (Hwang et al., 1994; Garcia-Closas and Lubin, 1999). On the contrary, the sample size cannot be calculated in case that the genotype frequency is unknown. In order to detect environmental factors in case-control studies including both subjects with and without the susceptible genotype, we had better have presumptions on the size (proportion) of individuals with the genotype and the OR among them. This paper aims to demonstrate minimal size of cases with the susceptible genotype to detect a significant environmental factor in case-control studies, as well as minimal required OR for individuals with the susceptible genotype.

Statistical Models

We recognized that there was a subgroup of cases with a genotype susceptible to an environmental factor. In order to calculate minimal detectable odds ratios of the environmental factor among those with the genotype ($OR_{susceptible}$), the following steps were made, as shown in Chart.

2.1. A proportion of exposed cases (P_1) producing a significant result in a case-control study with N_0 controls and N_1 cases was calculated based on a significance level (α), statistical power ($1-\beta$), and proportion of exposed controls (P_0), using the below conventional formula for a sample size calculation (Donner, 1984).

$$N_0 = \frac{[Z_\alpha \sqrt{(1+M)\,P\,(1-P)} + Z_\beta \sqrt{M\,P_0\,(1-P_0) + P_1\,(1-P_1)}\,]^2}{M\,(P_0 - P_1)^2}$$

where P is defined with $(P_0 + M\,P_1) / (1 + M)$, M with the ratio of N_1 / N_0, and Z_α and Z_β with the values derived from a normal distribution with mean=0 and variance=1 for a given significance level (α) and statistical power ($1-\beta$), respectively.

2.2. Odds ratio for whole subjects (OR_{whole}) was obtained by $P_1\,(1-P_0) / P_0\,(1-P_1)$.

2.3. P_1 was also defined with a weighted average calculated by $S\,P_x + (1 - S)\,P_0$, as shown in Fig 1. In this formula, P_x and P_0 were the proportions for the exposed in cases with and without the susceptible genotype, respectively. S was the size in proportion for cases with the genotype. It was assumed that the environmental exposure does not elevate the risk of disease for cases without the genotype. Accordingly, the proportion of the exposed among them was set to be the same as that among the controls, i.e., P_0.

2.4. S_{min} was defined as the S in case of P_x=1. It was the minimum of S, because P_x was the maximum at 1.

2.5. $OR_{susceptible}$ was calculated with $\{P_x\,(1-P_0)\}/\{P_0\,(1-P_x)\}$.

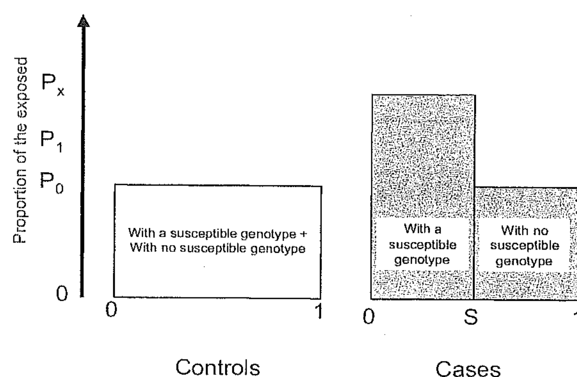


Figure 1. Proportions of the Exposed among Controls (P_0) and Cases (P_1). P_1 is the Average Proportion for Cases with a Susceptible Genotype (P_x) and Cases with no Susceptible genotype (P_0). The Area Surrounded by a Dotted Line is the Same as the Shadowed Areas. S is the Size in Proportion of Cases with a Susceptible Genotype.

Results

Since a large number of combinations exist, those with α=0.05 in a two-sided test (Z_α=1.96), $1-\beta$=0.80 (Z_β=0.842), and $N_0=N_1$ (M=1) were calculated as examples. Table 1 shows the calculated P_1, OR_{whole}, and S_{min}, when N_0 is fixed to be 200, 500, 1,000, or 2,000, and P_0 to be 0.05, 0.1, 0.3, 0.5 or 0.8. For example, a detectable P_1 was 0.638 for P_0=0.5 in a case-control study with 200 cases (N_1) and 200 controls (N_0), when α error of a two-sided test was 0.05 with an 80% of power. In case of P_1=0.638, OR_{whole} was 1.77, producing S_{min}=0.277 for infinite $OR_{susceptible}$. It indicates that an environmental factor cannot be detected in case that a high-risk genotype frequency is less than 0.277. Figure 2 depicts the relationship between S_{min} and N_0 for given P_0. The minimal size of cases with the genotype (S_{min}) increased with the proportion of the exposed in controls (P_0) and decreased with the number of controls (N_0).

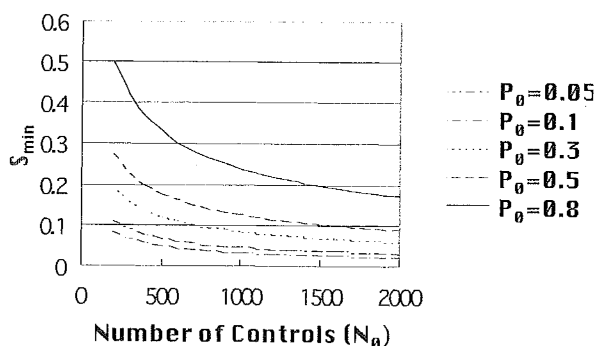


Figure 2. Minimal Size of Susceptible Cases Enabling to Detect a Significant Odds Ratio (S_{min}) According to Sample Sizes (N_0, in Case of $N_0=N_1$) and Proportion of the Exposed among Controls (P_0)

Table 1. Detectable Proportion of the Exposed among Cases (P_1), Odds Ratio for Whole Subjects (OR_{whole}), Minimal Size of Cases with a Susceptible Genotype (S_{min}) according to Number of Controls (N_0) and Proportion of Exposed Controls (P_0), under a Significance Level (α) = 0.05 for a Two-sided Test with Statistical Power (1-β) =0.8

N_0	P_0=0.05	P_0=0.1	P_0=0.3	P_0=0.5	P_0=0.8
			P_1		
200	0.130	0.200	0.435	0.638	0.900
500	0.096	0.160	0.384	0.588	0.866
1,000	0.081	0.141	0.359	0.563	0.848
2,000	0.071	0.128	0.341	0.544	0.834
			OR_{whole}		
200	2.84	2.25	1.79	1.77	2.25
500	2.02	1.71	1.45	1.43	1.62
1,000	1.67	1.47	1.31	1.29	1.39
2,000	1.46	1.32	1.21	1.19	1.26
			S_{min}		
200	0.084	0.111	0.192	0.277	0.499
500	0.048	0.066	0.120	0.176	0.330
1,000	0.033	0.045	0.084	0.125	0.239
2,000	0.022	0.031	0.059	0.088	0.171

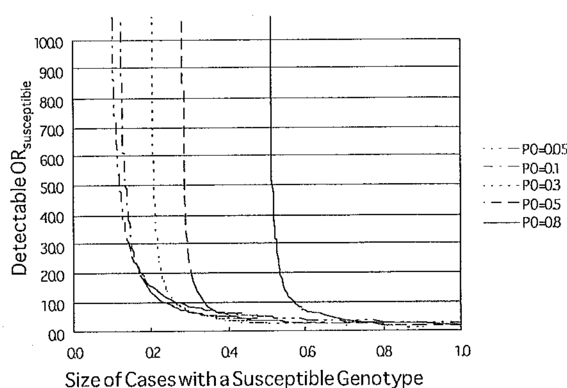
Figure 3 shows $OR_{susceptible}$ in a case-control study with 200 cases and 200 controls according to size of cases with the genotype (S) and proportion of the exposed controls (P_0). Since all the cases with the genotype were to be the exposed at S_{min}, the $OR_{susceptible}$ was infinite at S_{min}. In case of S > S_{min}, the $OR_{susceptible}$ decreased with S, and was equal to OR_{whole} at S=1. Figure 4 shows $OR_{susceptible}$ in case of P_0= 0.5 according to N_0 (=N_1). As N_0 was larger, $OR_{susceptible}$ was smaller in a given S. Table 2 lists the detectable $OR_{susceptible}$ according to S for different P_0 and N_0.

The above results can be used for the following examples. 1) When a case-control study has only 200 cases (N_1) and 200 controls (N_0), smoking can not be evaluable as a risk factor of male colon cancer in the following condition. Those with the susceptible genotype (S) are assumed to be 20% among the cases, and smokers are 50% among the controls (P_0). Table 1 provides S_{min} = 0.277 for N_0=N_1=200 and P_0=0.5, which is larger than the assumed S (0.2). 2) When a 30% of male colon cancer cases (S) have a genotype susceptible to smoking, $OR_{susceptible}$ more than 3.85 would be detected in a case-control study with 500 male cases (N_1) and 500 male controls (N_0), in an area where smokers are 50% among the male population (P_0) as indicated in Table 2.

Discussion

We know intuitively that risk factors affecting a small proportion of individuals may not be detected in a study, because of the effect dilution. Accordingly, even with a high penetrance, rare genotypes are not examined in association studies. As Shpilberg et al stated, "A twofold risk for 1000 exposed versus nonexposed people could be an average twofold risk for all 1000 exposed or a 20-fold risk for 100 exposed individuals" (Shpilberg et al., 1997). In case-control studies, however, there were no reference tables on the proportion of susceptible individuals. To date, several papers have been reporting required sample sizes for unmatched case-control studies to detect a gene-environment or gene-gene interaction (Hwang et al., 1994; Garcia-Closas and Lubin, 1999; Gauderman, 2002a; Gauderman, 2002b, Selinger-Leneman et al., 2003). But, their view is different from the present report. Tables and Figures presented in this paper provide useful information to avoid studies impossible to detect the significant results. The newly introduced concept, S_{min}, is an important measure when case-control studies are planned taking account of a susceptible subgroup in the study subjects.

In the present paper, the size of susceptible cases was



Figire 3. Detectable Minimal $OR_{subgroup}$ in a Case-control Study with 200 Cases and 200 Controls According to Size of Cases with a Susceptible Genotype (S) and Proportion of the Exposed among Controls (P_0)

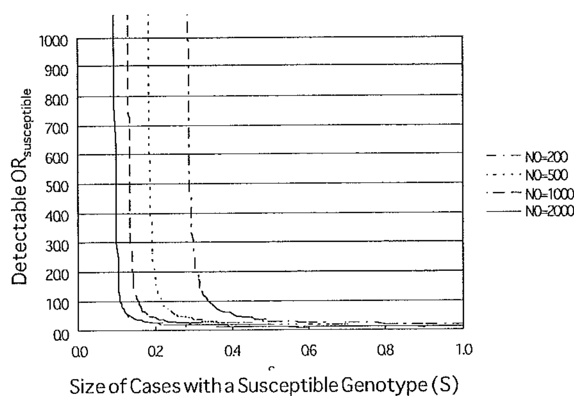


Figure 4. Detectable Minimal $OR_{subgroup}$ in a Case-control Study with Half of the Controls Exposed (P_0=0.5), According to Size of Cases with a Susceptible Genotype (S) and Number of Controls (N_0)

Table 2. Detectable OR for Individuals with a Genotype Susceptible to Environmental Factor ($OR_{susceptible}$) according to Size of Cases with the Susceptible Genotype (S), Proportion of Exposed Controls (P_0), and Number of Controls (N_0), under a Significance Level (α) = 0.05 for a Two-sided Test with Statistical Power (1-β) =0.8

N_0	S=0.1	S=0.2	S=0.3	S=0.5	S=0.7	S=1
P_0=0.05						
200	107	15.5	8.80	5.05	3.73	2.84
500	19.8	7.40	4.86	3.15	2.49	2.02
1,000	10.7	4.90	3.44	2.40	1.98	1.67
2,000	6.72	3.50	2.60	1.93	1.66	1.46
P_0=0.1						
200	N.E.	13.4	6.86	3.85	2.88	2.25
500	20.5	5.94	3.83	2.52	2.04	1.71
1,000	9.28	3.93	2.78	2.00	1.69	1.47
2,000	5.55	2.86	2.16	1.67	1.47	1.32
P_0=0.3						
200	N.E.	85.4	6.96	3.09	2.26	1.79
500	N.E.	6.00	3.22	2.05	1.69	1.45
1,000	18.6	3.42	2.30	1.67	1.46	1.31
2,000	5.81	2.40	1.82	1.45	1.31	1.21
P_0=0.5						
200	N.E.	N.E.	24.7	3.48	2.31	1.77
500	N.E.	15.9	3.85	2.09	1.67	1.43
1,000	N.E.	4.33	2.43	1.67	1.43	1.29
2,000	16.4	2.59	1.84	1.43	1.29	1.19
P_0=0.8						
200	N.E.	N.E.	N.E.	584	4.10	2.25
500	N.E.	N.E.	N.E.	3.43	2.12	1.62
1,000	N.E.	N.E.	5.85	2.14	1.65	1.39
2,000	N.E.	8.43	2.66	1.65	1.41	1.26

N.E.: ORsusceptible does not exist.

used, not of susceptible controls which represent the population without disease under study. Generally, the size of susceptible cases is larger than the size of susceptible controls ($S_{control}$). Although Tables and Figures could similarly be made using $S_{control}$, the size of susceptible cases (S) was adopted here. The S seems easier to be understood and estimated by clinicians, who are faced with patients.

In conclusion, this paper provided the useful figures when case-control studies on environmental factors interacting with genotypes are designed. These figures are applicable for OR of a genotype interacting with environmental factors, and also for gene-gene interactions to be derived from case-control studies based on high-throughput SNP analysis (Marnellos, 2003; McLeod and Yu, 2003).

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Chart for the Calculation Steps

1. Calculation of P_1 to obtain a significant result from given P_0, N_0, N_1, significance level, and statistical power.
2. Calculation of OR_{whole} from P_0 and P_1.
3. Calculation of P_x from P_0, P_1, and given S.
4. Calculation of S_{min} in case of P_x = 1.
5. Calculation of $OR_{susceptible}$ from P_0, P_x, and S.

N_0: Number of controls
N_1: Number of cases
P_0: Proportion of the exposed among controls
P_x: Proportion of the exposed among cases with a susceptible genotype
P_1: Proportion of the exposed among cases, which is defined with $S P_x + (1 - S) P_0$
S : Size (proportion) of cases with the susceptible genotype
S_{min}: The minimal S, i.e., S in case of P_1=1
OR_{whole}: Odds ratio for whole cases
$OR_{susceptible}$: Odds ratio for individuals with the susceptible genotype.

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Running headline

ENHANCED RT-PCR BY PREHEATING OF RNA IN CITRATE

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Improved RT-PCR amplification for molecular analyses with long-term preserved formalin-fixed, paraffin-embedded tissue specimens

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Running title: Enhanced RT-PCR amplification by preheating of RNA

Keywords: Preheating of RNA, archival formalin-fixed and paraffin-embedded tissue, citrate buffer, modification of RNA by formalin, RT-PCR amplification

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Abstract

Recently, in addition to DNA, RNA extracted from archival tissue specimens has become an invaluable source of material for molecular biological analysis. Successful amplification with PCR/RT-PCR is problematic when using amplicons of short size due to degradation of DNA or RNA. We established an improved method for efficient RT-PCR amplification of RNA extracted from archival formalin-fixed, paraffin-embedded tissue by the elimination of RNA modification and the restoration of RNA template activity. Namely, the preheating in citrate buffer (pH 4.0) of RNA extracted from long-term preserved tissue specimens resulted in significantly increased efficiency of RT-PCR.

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Retrospective analysis of archival tissue specimens is indispensable especially for studies of rare cancers or cancers associated with exposure to uncommon past events, such as Thorotrast treatment, nuclear power station accidents, or atomic bombings. In recent years, application of new molecular techniques including polymerase chain reaction (PCR) to analysis of archival tissue samples is anticipated to bring about better understanding of the molecular mechanisms of these cancers (Mies 1994). However, most surgical and autopsy tissue specimens have been preserved as formalin-fixed and paraffin-embedded blocks for long periods of time: DNA or RNA is often found to degrade under such conditions.

Extraction of DNA from formalin-fixed, paraffin-embedded tissue for PCR analysis has been well documented (Jackson et al. 1990; Forsthoefel et al. 1992; Frank et al. 1996). On the other hand, RNA was first extracted from formalin-fixed, paraffin-embedded tissue for northern and dot blotting analysis (Rupp and Locker 1988). Subsequently, many reports were made about extraction of viral or human cellular RNA from archival samples and successful amplification of extracted RNA (Weizäcker et al. 1991; Bresters et al. 1992; Finke et al. 1993; Koopmans et al. 1993; Goldsworthy et al. 1999; Masuda et al. 1999; Körbler et al. 2003; Byers et al. 2004). Recent reports showed that RNA extracted from formalin-fixed, paraffin-embedded tissue samples was also available for quantitative analyses of gene expression (Lehmann and Kreipe 2001; Specht et al. 2001; Cohen et al. 2002; Kim et al. 2003; Cronin et al. 2004).

Nevertheless, persistent demands have been made for further improvement in RNA extraction from "long-term preserved" tissue samples whose RNA significantly degraded being chemically modified. The problems facing this goal include degradation of RNA in tissue due to delay before fixation, prolonged fixation, or long-term preservation after fixation (Bresters et al. 1994; Cronin et al. 2004), low efficiency of RNA extraction because of cross-linking with proteins .

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(Finke et al. 1993; Park et al. 1996), and impaired reverse transcriptase reaction (Masuda et al. 1999) by formalin-induced modification (addition of mono-methylol to amino groups of four bases) of extracted RNA (Feldman 1973; Auerbach et al. 1977; Masuda et al. 1999). To overcome this problem, heat treatment of RNA prior to reverse-transcription has been proposed. For example, it was reported that the chemical modification of all four bases of RNA by fixation in phosphate-buffered formalin can be reversed to some extent by incubation in TE buffer (pH 7.0) at 70C for 1 hr, resulting in restoration of template activity of RNA in RT-PCR (Masuda et al. 1999).

While performing molecular analyses on cancer tissue specimens from atomic-bomb survivors that have been stored for several decades (up to 50 years), we have frequently encountered archival unbuffered formalin-fixed, paraffin-embedded specimens that are difficult to use for RT-PCR analysis. We found that significant degradation and chemical modification of RNA greatly affected RT-PCR amplification.

Removal of chemical modification from bases of RNA as well as significant reduction of amplicon size may be crucial for the enhanced availability of limited archival unbuffered formalin-fixed, paraffin-embedded tissue samples. Therefore, we examined whether the preheating of RNA extracted from archival unbuffered formalin-fixed, paraffin-embedded tissues enhances the efficiency of RT-PCR, along with determining optimal conditions for the RNA preheating. Application of this preheating technique to retrospective research is expected to enhance the availability of archival unbuffered formalin-fixed, paraffin-embedded tissue specimens for molecular analysis that have been stored for several decades and that have functioned as a source for histological evaluation, and to allow better understanding of molecular characteristics of various diseases.

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Material and methods

Tissue

Five archival unbuffered formalin-fixed, paraffin-embedded thyroid cancer tissue samples for in-house control were used in this study. All samples were preserved at room temperature for 19-21 years. After deparaffinization of 5 μm sections by Hemo-De (Fujisawa Yakuhin Kogyo; Osaka, Japan) and staining with methylgreen (Sigma-Aldrich; St. Louis, MO), cancerous regions (about 2~3 x 3 mm) were isolated using a laser microdissection system, Leica AS LMD (Leica; Wetzlar, Germany). All cancerous regions microdissected from 6 ~ 8 successive tissue sections were combined for RNA extraction.

RNA extraction and measurement

RNA was isolated from microdissected tissue using the High Pure RNA Paraffin kit according to the manufacture's instructions (Roche Diagnostics; Basel, Switzerland), with some modifications. Briefly, microdissected tissue was digested with proteinase K at 55C overnight, followed by DNase I treatment. After the lysate was purified by High Pure filter, RNA was eluted twice with 100 μl of RNase-free water. RNA was then precipitated by ethanol in the presence of 2 μl of ethachinmate (Nippon Gene; Tokyo, Japan) as a carrier and resuspended in 30 μl of RNase-free water. The concentration of RNA was measured by absorption at 260 nm with a spectrophotometer, Gene Spec III (Hitachi; Tokyo Japan). The quality of extracted RNA was measured by electrophoresis on 1.5% or 3.0% native agarose gel and 2.5% formaldehyde agarose gel.

Heat treatment of RNA

About 150 ng of total RNA were heated in 250 μl of 10 mM citrate buffer with various pH values ranging from 3 to 6.5 at 70C for a number of different time-periods. Preheating in 250 μl

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of 10 mM sodium borate with various pH values (pH 6.5-10) or 10 mM TE buffer (pH 7.0, 7.5 and 8.0) was similarly carried out. After preheating, RNA was precipitated by ethanol in the presence of ethachinmate as carrier and dissolved in RNase-free water to arrive at a final concentration of 10 ng/μl.

cDNA synthesis

One hundred nanograms of total RNA and 50 pmol/μl of random primers (9 mer) were heated in 11 μl of RNase-free water at 65C for 10 min and chilled in ice water. A mixture consisting of 4 μl of 5 X RT buffer, 2 μl of 20 mM DTT, 1 μl of 10 mM dNTPs, and 1 μl of RNase Inhibitor (20 U/μl, Takara; Tokyo, Japan) was added to RNA solution and incubated at room temperature for 5 min. After addition of 1 μl of Rever Tra Ace (100 U/μl, Toyobo; Osaka, Japan), a reaction mixture was incubated at 42C for 60 min and at 70C for 15 min.

Detection of expression of breakpoint cluster region (*BCR*) and *N-ras* genes by RT-PCR

RT-PCR was performed in a 25 μl volume containing 1X PCR buffer, 200 μM each of dATP, dCTP, dGTP and dTTP, 1.5~3.0 mM $MgCl_2$, 0.4 μM of each specific primer, 0.5 U Platinum Taq DNA polymerase (Invitrogen; USA), and 2 μl of cDNA from the previous RT reaction. Primary denaturation (95C for 3 min) and final extension (72C for 5 min) were the same for each RT-PCR reaction, all of which were subjected to 40 cycles of amplification consisting of 95C for 30 sec, 55~60C for 30 sec and 72C for 30~45 sec for *BCR*, and 95C for 30 sec, 52~55C for 30 sec and 72C for 30~45 sec for *N-ras*. For positive and negative controls of RT-PCR in Figures 3 - 7, cDNA derived from human thyroid cancer cell line (8505C) and H_2O were used as templates, respectively. Five microliters of the reaction mixture were run on an 8% acrylamide gel and visualized with ethidium bromide. In each experiment, it was confirmed that all target bands were the real ones by digestion of restriction enzyme, which existed within each amplified target

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fragment. Since all the primer sets used were designed to locate in two different exons, no amplification of RNA without RT was observed. The effects of preheating on RT-PCR efficiency were examined by amplifying the fragments of different sizes in the *BCR* gene (8 different sizes, 61 bp, 94 bp, 127bp, 152bp, 175 bp, 222 bp, 250 bp, 275 bp) and the *N-ras* gene (7 different sizes, 61 bp, 98 bp, 121 bp, 148 bp, 199 bp, 221 bp, 250 bp). The semi-quantification of each PCR product was made by measurement of the intensity of each band using Kodak 1D Image Analysis Software.

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Results

RNA extracted from unbuffered formalin-fixed, paraffin-embedded tissue

We extracted RNA from five different archival unbuffered formalin-fixed, paraffin-embedded thyroid cancer tissue specimens, as described in Materials and Methods. An image of electrophoresis of these RNA is shown in Figure 1. They appeared as smears on agarose gel, with no ribosomal bands observed in any of the samples. The range of smeared RNA differed slightly among five archival tissue specimens. A majority of the smeared RNA sample used in Figures 3-6 ranged from about 70 bases to 100 bases (Figure 1, lane 2). Other RNA samples used in Figure 7 (C and D) ranged from about 70 bases to 200-300 bases (Figure 1, lanes 3 and 4). The size of RNA from the remaining two tissue samples was the intermediate among the other three samples.

Temperature in preheating of RNA

At first, we tested the effects of incubation at different temperatures on RNA stability using intact RNA prepared from human thyroid cancer cell lines, because it is hard to evaluate the preheating effect using already degraded RNA extracted from formalin-fixed, paraffin-embedded tissue specimens (Figure 2). Preheating of RNA at more than 80C for 30 min in H_2O resulted in vigorous degradation. Considering our result and a report by Masuda et al. (1999), we set preheating temperature at 70C in this study.

The effects of pH in preheating of RNA on RT-PCR amplification

Using the RNA with the worst efficiency of RT-PCR amplification among the five archival tissue samples, we examined the effects of preheating on RT-PCR efficiency by amplifying the fragments of different sizes in the *BCR* gene and the *N-ras* gene (Figure 3). Sixty-one bp fragments in the *BCR* and *N-ras* genes were detected in the RNA not undergoing preheating,

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although the intensity of the bands was weak compared with that in the RNA with preheating (Figure 3). Preheating of RNA in 10 mM citrate buffer at pH 3 - 5 at 70C for 30 min made it possible to amplify 94 bp and 127 bp fragments in the *BCR* gene and 98 bp fragments in the *N-ras* gene; these same fragments could not be amplified by RT-PCR without undergoing preheating (Figure 3). Preheating of RNA in citrate buffer with pH of around 4 was the most effective method in the RT-PCR amplification of the *BCR* and *N-ras* genes. To determine optimal pH, we further investigated in detail the effects of pH on RT-PCR amplification using preheated RNA. As shown in Figure 4, RNA treated with around pH 4.0 showed the most efficient RT-PCR amplification of both the *BCR* and *N-ras* genes among citrate buffers with different pH-values (ranging 3 - 5) and TE (pH 7.0). We also examined the effect of pH range (6.5 - 10) on RT-PCR amplification using 10 mM sodium borate solution and TE. Among buffers ranging from pH 7.0 to pH 8.0 (TE buffers with pH 7.0, 7.5 and 8.0, and borate buffer with 8.0), little difference was found in the effect of preheating with these buffers at a concentration of 10 mM (data not shown). The effect of preheating decreased with increased pH, and adverse effect was observed in RNA treated with pH 9.0 or 10.0 (data not shown). Little difference was found in efficiency of RT-PCR amplification enhanced by preheating of RNA between citrate buffer (pH 6.5) and sodium borate (pH 6.5) (data not shown).

The effects of buffer concentration in preheating of RNA on RT-PCR amplification

We examined the effects of different buffer concentrations on RT-PCR amplification. In both *BCR* and *N-ras* target genes, when RNA was heated with 50 mM citrate buffer at 70C, even 61 bp fragments were hard to detect, a lower efficiency of RT-PCR amplification than amplification without preheating (Figure 5). On the other hand, treatment with 10 mM citrate buffer resulted in the greatest efficiency of RT-PCR amplification among concentrations investigated (Figure 5).

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The effects of preheating time of RNA on RT-PCR amplification

We examined the effects of preheating time on RT-PCR amplification. As shown in Figure 6, preheating in citrate buffer (pH 4.0) at 70C for 45 min was the most effective method for RT-PCR amplification. Thus, the efficiency of RT-PCR amplification depends on pH, concentration of buffer, and incubation time. In the other four archival thyroid tissue samples we also examined whether preheating of RNA in citrate buffer (pH 4.0) at 70C for 45 min resulted in enhanced efficiency of RT-PCR amplification. We found that the longer fragments in the preheated RNA could be amplified in all cases compared with the non-treated RNA, although the amplified fragment sizes differed among these thyroid tissue samples (Figure 7). Increased efficiency of RT-PCR amplification, therefore, was observed in all five unbuffered formalin-fixed, paraffin-embedded thyroid tissue samples through the preheating of RNA in citrate buffer (pH 4.0).

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