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National Research Program for Genomic Medicine
National Science Council, the Executive Yuan, ROC.

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Linkage Analysis of Susceptibility Genes for Bladder Cancer in Multiplex Families (3/3)

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› p e¥ D ø⁄H(Principle Investigator)¡ G ‟ ¥ ..(Hung-Yi Chiou)

ı ƒ ‡ ƒ(Institution)¡G¥ x ¥ _ ´ ˙ ⁄ j ⁄‰ƒ@‰ˆ¥˝ ˙¤t

(School of Public Health, Taipei Medical University)

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National Research Program for Genomic Medicine
National Science Council, the Executive Yuan, ROC.
Progress Report—Research Project

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Program Classification:

☑ **Genomic Medicine** ☐ **Bioinformatics**

☐ **Proteomics & Structural Genomics** ☐ **ELSI**

Project Number: **91GMP012-6** (› p ÷s), „

NSC Funding Number

NSC93-3112-B038-002 (93 ƒ ~ « Œ‹„ wfi ‰ ֆ„)

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	(in English) › ᢦ Linkage Analysis of Susceptibility Genes for Bladder Cancer in Multiplex Families
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Principle Investigator ›p e¥D«ø⁄H	(in Chinese) ⁄⁄⁄ ‟ ¥ …
	(in English) › ᢦ Hung-Yi Chiou

FY	2002	2003	2004	Total
Budget				

(in NT dollars: 1USD = 34 NTD)

Signature of the PI¡G______________**Date**¡G__12/24/2003____

Part B. Progress Report of Component Projects

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Part B. Progress Report

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B2. Progress Report
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  B2b. Studies & Results
  B2c. Personnel
  B2d. Projected Timeline
  B2e. Publications (Optional)
  B2f. Patents (Optional)

B3. Request for Modifications of the Project (Optional)

You do not have to finish this section except your budgets or items of requests are modified.

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  B3a. Background & Statement (including literature cited)
  B3b. Summary Budget Requested
  B3c. Postdoctoral Fellows Requested
  B3d. Detailed Budget for Personnel
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  B3j. Biographical Sketches of New Personnel

PROGRAM PROJECT: Component Project ___6__ (☜‰ – ¶ æ ⁄ J ⁄ ɫp) e‰s,„

B2. Progress Report

B2a. Specific Aims

Please state the overall goals of the project, and specific aims, as reviewed and approved by the Study Section and actually awarded. If these specific aims as actually funded did not differ in scope from those actually pursued during the grant period, and if the aims have not been modified, state this. If they have been modified, give the revised aims and the reasons for the modifications.

The overall goals of this subproject are to identify and differentiate the major susceptibility gene(s) for arsenic-induced and non-arsenic-induced bladder cancers through the linkage analysis of genetic markers in members of multiplex families. This subproject will also set up a Genetic Epidemiology and Biostatistics Supporting Core for this program project. It will include following specific aims: 1) the ascertainment of multiplex families through the telephone or home-visit interview, based on a family history questionnaire, of bladder cancer cases reported to the national cancer registry and cases diagnosed and treated in collaborative medical centers; 2) the recruitment of probands and families members through home-visit personal questionnaire interview and biospecimen collection; 3) the purification, depository and inventory of DNA samples in central biospecimen bank; 4) the typing of genetic markers on chromosomes in which loss or gain have been observed through previous comparative genomic hybridization and loss of heterozygosity studies; 5) the mapping of susceptibility gene(s) through linkage analysis of multiplex family data for bladder cancer cases in arseniasis-endemic and non-endemic areas; 6) the examination of the effects of possible candidate genetic marker(s) and their synergistic interactions with environmental factors on bladder cancer; and 7) the establishment of Genetic Epidemiology and Biostatistics Supporting Core to provide methodological support to other subprojects. We do not modified any specific aims of this component project. Base on experience of the first year, we have confidence of accomplishing these specific aims. However, the total number of multiplex families will be less than the proposed number because the modified budgets raised in the initial time of the second grant year was not proved. Based on our study capacity, we expect a total of 60 multiplex families will be recruited during three year grant period.

PROGRAM PROJECT: Component Project 6 (☜‰–¶æ⁄J⁄l›p é‰s, „)

B2b. Studies and Results

Describe the studies directed toward the specific aims during the current grant period and the results obtained. Indicate the extent to which the work accomplished has successfully met the specific aims. Include negative results. If technical problems were encountered in carrying out this project, describe how your approach was modified.

Background and Significance

Our previous studies have shown that capability of arsenic methylation is associated with the risk of arsenic-induced skin cancer (12). Null genotypes of glutathione S-transferase (GST) M and T1 and variant genotype of GST P1 have been found to increase the risk of arsenic-induced skin cancer (13). Whether the genotypic and phenotypic polymorphisms of GSTs and other enzymes related to arsenic methylation may modify the risk of arsenic-induced TCC remain to be elucidated. Furthermore, genetic polymorphisms of some DNA repair enzymes have been observed to be associated with the risk of arsenic-induced skin cancer (14). It is also important to assess whether the DNA repair enzymes, either their genotypes or gene expression profiles, are related to the risk of arsenic-induced bladder cancer. The existence of genetic susceptibility to arsenic-induced bladder cancer may be explored through the microarray analysis of differential expression profiles and single nucleotide polymorphisms of related genes. It may also be investigated by the genetic epidemiological study on familial TCC through linkage analysis.

Tobacco smoke is another important etiological agent of urinary bladder cancer in Taiwan. It plays an important role in the induction of urinary bladder cancer in non-arseniasis-endemic areas. The tobacco-related bladder cancer risk is modified by the genetic polymorphisms of N-acetyltransferase 1 and 2 (15). The comparison of toxicological genomic characteristics of TCC induced by tobacco and arsenic may help the elucidation of the arsenic-induced carcinogenicity. This program project is organized to study the difference in toxicological genomic characteristics between TCC induced by arsenic and tobacco smoke.

Due to the lack of animal models for arsenic carcinogenicity, arsenic is a unique carcinogen in humans. Therefore, the high prevalence of urinary TCC in arseniasis-endemic area in Taiwan is a unique chance to investigate the carcinogenicity of arsenic. Since cancer development is mainly attributable to environmental factors and genes, it is of fundamental importance to understand the interaction between environment and genes. In this PPG, epidemiological components will establish the association of environmental factors and incidences of urinary TCC. Molecular, cellular and genetic components will identify the genetic factors involved in formation of urinary TCC. The joint efforts of epidemiological and molecular genetic studies will successfully dissect the interactions between environmental factors and genes. Novel findings of this PPG will allow us designing new strategy for prevention, diagnosis, and treatment of urinary TCC.

B2b. Studies and Results **(Continue)**

Though arsenic is a human carcinogen, there is no good animal model for the carcinogenicity of inorganic arsenic. Arsenic is inactive or extremely weak to induce gene mutations at specific loci (1,16). The modes of action for arsenic-induced carcinogenicity might include the induction of chromosome abnormality, inhibition of DNA repair, induction of oxidative stress, and increase of cell proliferation (17).

Inorganic arsenic has several genotoxic effects including the induction of changes in chromosome structure and number, increases in sister chromatid exchanges and micronuclei, gene amplification, cell transformation, aneuploidy, and chromosome-type chromosome aberration (1,17-22). The role of inorganic arsenic in the carcinogenesis has also been hypothesized as a co-carcinogen such as promoter or progressor rather than an initiator (23,24). However, the evidence is far from adequate to draw a definite conclusion on the exact mechanism of inorganic arsenic to induce various cancers in humans.

Most of the genetic variants which have been studied using the design of case-control study were only associated with a modest increased genetic risk for developing cancer, although the magnitude of their attributable risk is large because there are quite frequent in the population. Many rare cancer syndromes due to a mutated gene which confer very high risk of cancer have been well-documented, including the rare Li Fraumeni symdrom in which persons inhriting a gerlime mutation in the p53 gene are at almost 100 % risk of breast caner and other cancers by the age of 60 years (25,26), the familial adenomatous polyposis symdrome associated with mutation in the APC gene (27), and the breast-ovary cancer syndrome associated with an inherited mutation in BRCA1 (28).

Human genome sequence have been cloned successfully by “Human Genome Project”. A total of 3×10^5 of an estimated $3\text{-}10 \times 10^6$ single nucleotide polymorphisms which distinguish individuals and their disease traits and risk will be identified by 2002 (29). Classical family, twin and adoptee studies have shown substantial heritabilities for many disease traits, but except in rare instances the pattern is polygenic rather than monogenic. Knowledge and technology have become sufficient to enable molecular geneticists to study megaphenic disorders in single families, with the exceptation of isolating ‘the genes’ understanding the pathology and deriving clinically applicable test of status (30,31).

For polygenic traits, knowledge is sufficient to initiate tests of hypotheses, but the technology is yet insufficient to measure the contribution of genetic diversity to disease liabilities. Linkage analysis of multiplex family has an important role in genetic epidemiology because it identifies a biologic mechanism for transmission of a trait or disease (31). A large family displaying a clear-cut segregation pattern for a disease is examined at polymorphic sites representing each part of each chromosome (30)

. Recently, most sites of cancer has been recognized as multigene disorders. It means that many regions of each chromosome have been examined intensively to identify susceptible genes and then to differentia their functions on risk of various cancers. With a availability of a fairly complete human genome sequence, this approach is reducing to searching the sequence for the possible culprit gene on criteria such as its tissue expression pattern, apparent function predicted from sequence, etc. For example, haemochromatosis was shown by positional cloning to be attributable to an HLA-related genes and it has immediately been possible to examine the relationship between genetic and iron status diagnostics, population prevalence of mutations, and prevalence of HFE mutations in haemochromatosis-associated disease groups such as diabetics (32). In addition, given the widespread roles of iron, for various phenotypes and investigation of HFE interactions in other disorders such as porphyries, haemoglobinopathies and coronary disease and environmental variables such as diet, lead poisoning and infections, is now proceeding to explore gene-environment and gene-gene interactions through epidemiological design (33-35).

The transformation of a normal cell into a malignant cell is a multistep mechanism, which involves various alterations on the molecular and genetic level. These molecular alterations occur spontaneously or are induced by carcinogens. As in most other malignancies the development of bladder cancer is caused by the accumulation of various molecular changes. The expression of oncogenes (ras, erbB-2 and EGF receptor), tumor-suppressor genes (Rb, p53),
cell-cycle genes (p15, p16) and DNA-repair genes is altered mostly by mutation or chromosomal aberration. Loss of heterozygosity of chromosome 9p and 9q has been shown to be a crucial event in the transition of normal urothelium to papillary transitional cell carcinoma while p53 is primarily involved in the development of carcinoma in situ (36). A study based on 44788 pairs of twin from Sweden, Denmark, and Finland showed that heritability was estimated to account 31 percent of the variation in susceptibility to the risk of bladder cancer. It was implied that genetic component might not be ignored in the carcinogeneicity of bladder cancer (37).

In this study, by the aid of linkage analysis, we plan to map the susceptibility gene(s) through multiplex family data of bladder cancer cases from arseniasis-endemic and non-endemic areas, to differentiate the difference between gene profiles of residents from arseniasis-endemic and non-endemic areas and to clarify the genetic and functional changes that make a person affected with arsenic-induced bladder cancer.

¢„ Multiplex families ascertainment

A total of 730 urothelial carcinoma (UC) consenting participants have been recruited. Among them, 131 consenting participants from Taiwan University Hospital, 469 consenting participants from Chi-Mei Hospital and 130 consenting participants from Chia-Yi Christian Hospital. An informed consent will be obtained from each participant for the collection related study information. through personal interview in centers for outpatient and inpatient by well trained interviewer. Risk factor questionnaire (RFQ) was used to collect risk factors of UC including cigarette smoking, alcohol drinking, well water consumption, nutrition status, and usage of dye and drug. Family history questionnaire (FHQ) was used to obtain disease history of various cancers especially for UC and chronic diseases including diabetes mellitus (DM) and hypertension. Flow chart for multiplex families recruitment was shown in Figure 1.

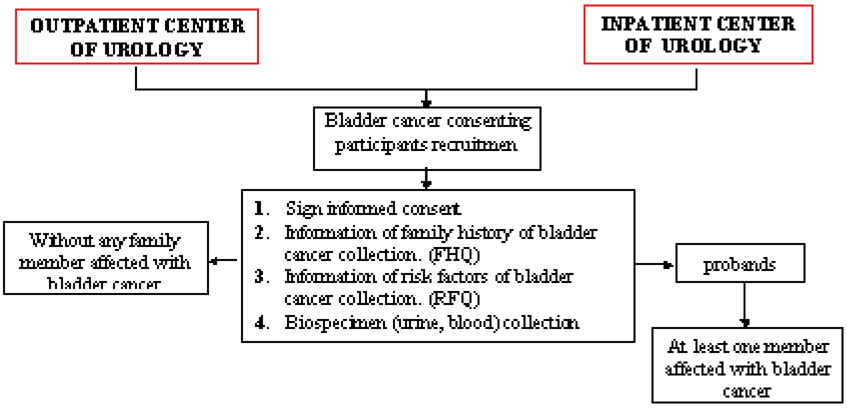
OUTPATIENT CENTER OF UROLOGY
INPATIENT CENTER OF UROLOGY
Bladder cancer consenting participants recruitmen
1. Sign informed consent
2. Information of family history of bladder cancer collection. (FHQ)
3. Information of risk factors of bladder cancer collection. (RFQ)
4. Biospecimen (urine, blood) collection
Without any family member affected with bladder cancer
probands
At least one member affected with bladder cancer

Figure1. Flow-chart for Multiplex Family Astertainment (Phase I)

¢″. Recruitment of proband and families members

A total of 21 probands including 4 from nonarsenic-exposed area and 17 from arsenic-exposed area , we recruit their first degree relatives with UC have been ascertained through FHQ. Now we have mailed invited letters to family members of probands to invite them to participate this study. Among consenting family members, we decided to recruit six members of each multiplex family including parents, spouse, sibling, and offspring. Questionnaire interview and biospecimen (blood, buccal cell, urine) collection will be executed either in outpatient center of collaborative medical center or household of consenting family members. The flow chart of multiplex families recruitment was shown in figure 2. Socio-demographic characteristics of probands and their first -degree relatives were shown in Table 1 and 2. Pedigrees of eight additive multiplex families newly recruited were illustrated in Figure 3-1 to Figure 3-8.

B2b. Studies and Results **(Continue)**

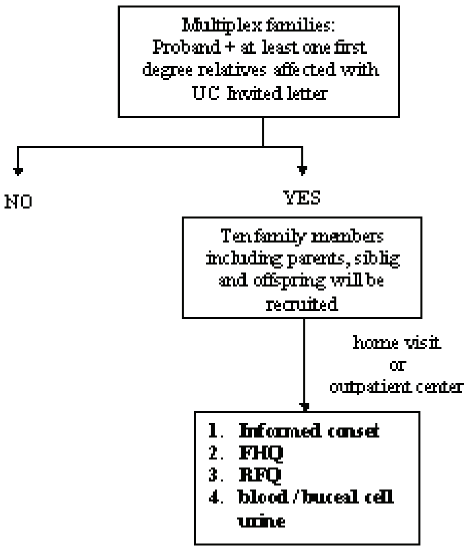
Multiplex families:
Proband + at least one first degree relatives affected with UC Invited letter
NO
YES
Ten family members including parents, siblig and offspring will be recruited
home visit
or
outpatient center
1. Informed conset
2. FHQ
3. RFQ
4. blood / buccal cell urine

Figure2. Flow-chart for Multiplex families recruitment (Phase II)

Initially, among consenting family members, we decided to recruit six members of each multiplex family including parents, spouse, sibling, and offspring. However, after trying to invite these relatives to participate in this study , about half of them can not be completely interviewed or even refused to be recruited. Reasons for not recruiting or not completing the questionnaire and biospecimen collection were: “no contact with relatives,” “too much effort,” “too emotional,” “too complicated to retrieve all data on relatives” ,“no permission from relatives to give information.”, ”expired address and telephone number” ,“too far or no one can take them to the hospital”, and especially, some probands already died before we have recruited them. In order to overcome these difficulties, we devoted to several strategies and did more intensive efforts to contact them for interview to achieve the goal as followed: [1] outreach to interview those unable to be the hospital ; [2] recheck their medical records and get the correct address and telephone number; [3] instead of face-to-face interview, we try to get the information by postal questionnaire. [4] give some kinds of presents or additional information and a reply card to encourage them to participate in this study.

[5] recruit more collaborated hospitals to increase the number of multiplex families and first-degree relatives; [6] subsequent telephone calls were made for additional information of uncompleted questionnaires; [7] refer the first-degree relatives living in the catchment area of our collaborated hospital to receive the face-to face interview; [8] link medical records and cancer registry database to identify new UC cases and update associated mortality information.

Table 1. The distribution of age and sex 21 UC probands

Family ID	Sex	Age (year)
NTU10222	M	61
NTU10263	F	67
NTU10310	M	61
NTU10264	M	66
CM1010	M	71
CM1012	M	85
CM1071	M	57
CM1059	F	62
CM1123	M	73
CM1356	F	96
CM1352	M	64
CM1355	F	65
CM1485	M	58
CM1271	M	73
CM1272	F	48
CM1402	M	60
CY1001	F	50
CY1032	F	85
CY1000	F	64
CY1056	F	45
CY1131	F	58

mean age:65.2 years old; sex ratio (M:F)=11:10

Table 2. The distribution of first degree relatives among 21 UC multiplex families

		First-degree relatives					
Family ID	**members**	**Father**	**mother**	**brother**	**sister**	**son**	**daughter**
NTU10222	11	1(1)	1	4	4	1	0
NTU10263	10	1(1)	1	1	2	4	1
NTU10310	6	1(1)	1	0	0	3	1
NTU10264	12	1(1)	1	1	5	3	1
CM1010	9	1(1)	1	2	1	3	1
CM1012	14	1	1	2(1)	2	2	6
CM1071	14	1	1	6(1)	2	2	2
CM1059	10	1	1(1)	3	3	1	1
CM1123	9	1	1(1)	2	1	1	3
CM1356	14	1	1	1	3	4(1)	4
CM1352	10	1	1	3	2	2	1
CM1355	11	1	1	3(1)	2(1)	1	3
CM1485	15	1(1)	1	7	3	1	2
CM1271	10	1	1	0	1	4(1)	3
CM1272	6	1	1	3(1)	0	1	0
CM1402	11	1(1)	1	3	1	1	4
CY1000	9	1	1	1(1)	1	4	1
CY1001	8	1	1	1(1)	2	1	2
CY1032	17	1	1	3	6(1)	1	5
CY1056	7	1	1	2(1)	1	0	2
CY1131	10	1	1	2	2	3(2)	1

*** Number of first-degree relatives affected with UC are shown in parenthesis**

Figure-3.1

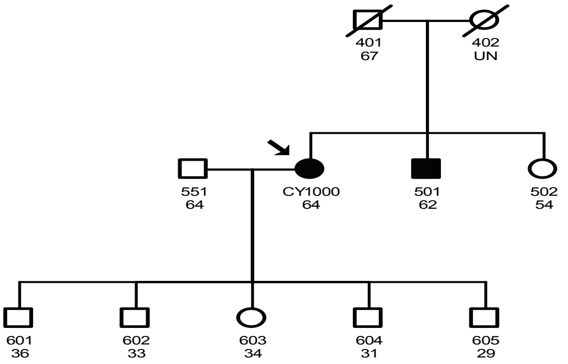
401
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Figure-3.2

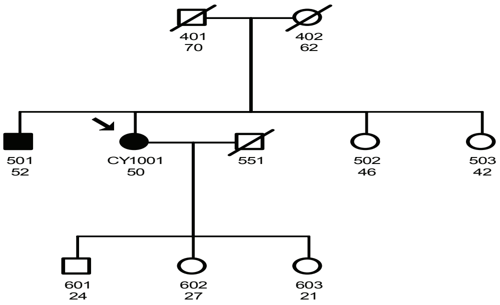
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Figure-3.3

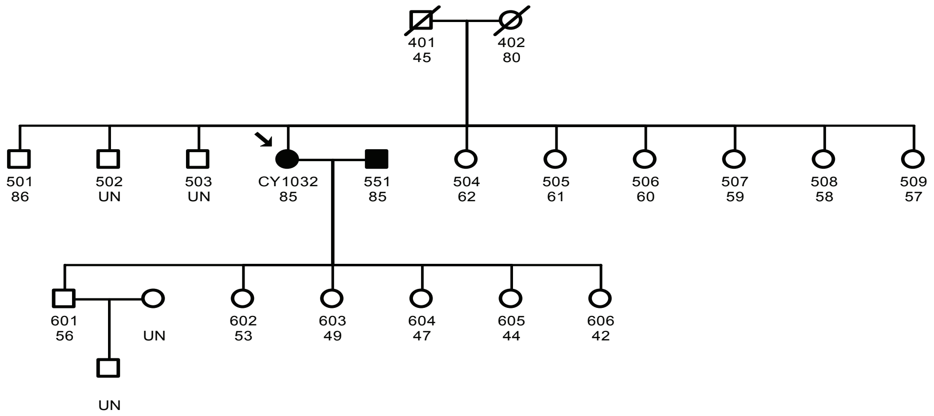
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Figure-3.4

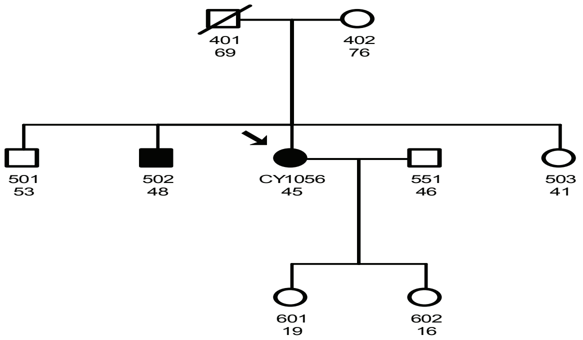
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Figure-3.5

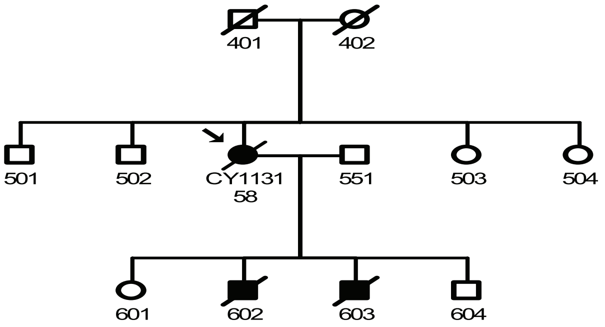
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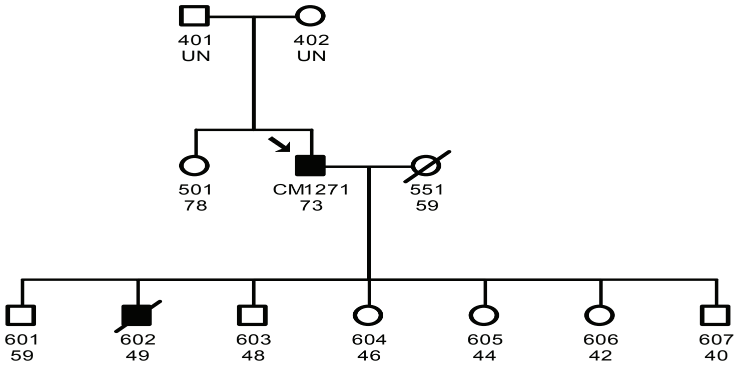
Figure-3.6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CM1271
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B2b. Studies and Results (Cont.)

Figure-3.7

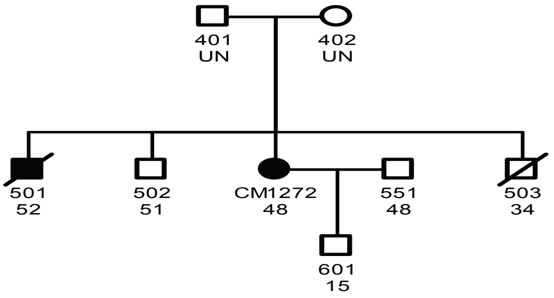
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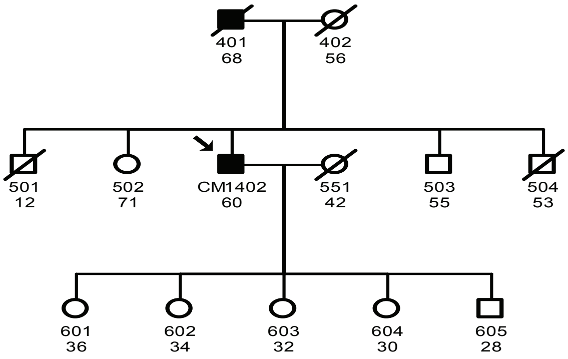
Figure-3.8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CM1402
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28

B2b. Studies and Results (Cont.)

¢»Risk Ratios for Relatives

In human genetics and genetic epidemiology, risk ratios can assume a number of different forms, including risk ratios for relatives, for candidate genes, and for genetic markers. The goal of many genetic studies is to quantify the risk of disease occurrence associated with particular genetic factors. Evidence for familial aggregation, which is the tendency of disease to cluster in families, provides a rationale for subsequent genetic studies intended to assess particular genetic factors or to search for disease susceptibility genes.

The most important epidemiological parameter is the relative risk , λ_R, defined as the recurrence risk for a relative of an affected person divided by the risk for the general population prevalence *K* (40). The subscript R denotes the type of relation; for example, λo and λs are the risks to offspring and sibs, respectively. The magnitude of λ_R is related to the degree of concordant inheritance for genetic determinants in affected relative pairs and thus is related to the ease or difficulty of genetic mapping, as shown by Risch (41-43)

Table 4. The estimated Recurrence Risk Ratio(RRR) of 730 urothelial cell carcinoma (UC) consenting participants

Type of relatives	number (affected)	siblings only(λs)	all relatives (λ_R)
Parents	1460 (9)		
Sibling	3650 (9)	8.2	
Offspring	2920 (5)		
All relatives	8030 (23)		9.5

* The population prevalence was estimated as 30/100,000.

Genetic mapping is much easier for traits with high λ (for example, $\lambda_S > 10$) than for those with low λ (for example, $\lambda_S < 2$). The tentatively estimated λ_S found in this study were 8.2 for siblings only and 9.5 for all relatives. Compared with other diseases as illustration of the range, $\lambda_S \approx 500$ for cystic fibrosis; 15 for type I diabetes [of which a factor of 3 to 4 is attributable to concordance at the human leukocyte antigen (HLA) complex]; 8.6 for schizophrenia; and 3.5 for type II diabetes (44-45). For a quantitative phenotype, a similar measure is the heritability of the trait .These values underestimate the population risk and therefore overestimate familial aggregation. A mathematical foundation for the choice of the general population risk as the reference value for calculating the sibling recurrence-risk ratio as a tool for mapping genes for complex diseases was clarified by the work of Risch in the early 1990s [46-47]. As λ_S is used to design studies and to estimate the power to detect genes for complex human disease, it must be determined [48–49].

IV. DNA purification, depository and inventory in central biospecimen bank

10 ml blood was obtained from each consenting participant. Total genomic DNA was purified using Viogene Blood and tissue Genomic DNA Miniprep System from buffy coat. In core of biospecimens bank, each DNA sample was deposited at -70¢J refrigerators and also be inventoried in computer files. Table 3 showed number of RFQ, FHQ, blood sample and urine sample collected from consenting participants through Taiwan university Hospital, Chi-Mei Hospital and Chia-Yi Hospital.

Table 3. The Questionnaires and biospecimens of consenting participants

Item	Taiwan University Hospital		Chi-Mei Hospital		Chia-Yi Christian Hospital	
	N	(%)[a]	N	(%)[a]	N	(%)[a]
RFQ	131	(18)	469	(64)	129	(18)
FHQ	131	(18)	393	(54)	129	(18)
Blood	92	(13)	381	(52)	130	(18)
Urine	110	(15)	38	(5)	130	(18)

a: percentage of 730 consenting participants

Although, the collection of questionnaire and biospecimen was not comprehensive, we still try to do more efforts to increase the comprehensive rate. Some cases or their relatives are difficult to get appropriate volume of blood or urine, we use the buccal swab to collect buccal cells instead of blood samples. In another method, because patients will return to the hospital to have a routine examination, we mentioned interviewers and staffs of the Laboratory Science Department to collect un-recruited samples in this routine process.

At the same time, we extracted DNA from buffy coat or buccal cellls from biospecimen bank. If the patients died for several years ago, we try to check the tissue bank of the collaborated hospitals to get appropriate amounts of tissues for DNA extraction. Then we will performed PCR-RFLP to analyze the genotype of several UC associated susceptible genes such as NQO1, iNOS, EGFR.

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B2c. Personnel

Summarize the personnel involved in the project during the grant period. List the personnel in accordance to the following categories: (1) senior investigators, including visitors; (2) postdoctoral fellows; (3) graduate students; (4) technicians; and (5) other research assistants. Specify for each individual the period of involvement and the percentage commitment of effort.

Name		Position Title	Education Degree	% of effort	Job Description or Responsibilities
In Chinese	In English				
" ¥…	Hung-Yi Chiou	Professor	Ph.D.	30%	Director of the component project
ǂ fl «⁄fl	Chien-Jen Chen	Professor	Sc.D.	5%	Consultant of the component project
" ⁄†»	Wen-Hsiang Chiou	Associate Professor	MD, Ph.D.	10%	UC patients diagnosis and recruitment
⁄ ₩§»	Iuang-Hung Wang	Doctor student	MPH	40%	Set-up of flow-chart of UC patient recruitment and data management
fi } — ~	Yi-Hwa Hsu	Full-time research assistant	MPH	100%	Questionnaire interview and biospecimens collection
¿ ‰ ″‚R	Jui-Chi Hsiao	Full-time research assistant	BS	100%	Questionnaire interview and biospecimens collection
` ´ ỷª‚	Yi-Chen Hsieh	Master student	BS	40%	DNA purification, depository and inventory

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B2d. Projected Timeline

Provide a reasonable timetable for the execution of the work outlined in the project. Highlight appropriate milestones that you might use to target the studies. Indicate technical hurdles that might slow down the execution of the work and discuss any contingencies that you have or might have built in the research plan in anticipation of these difficulties. Do not exceed one page.

Component projected timetable (second year)

The second Year **Month Order** **Item**	**1st Month**	**2nd Month**	**3rd Month**	**4th Month**	**5th Month**	**6th Month**	**7th Month**	**8th Month**	**9th Month**	**10th Month**	**11th Month**	**12th Month**	**Remark**
Recruitment of more collaborative medical centers	━	━	━	━	━	━	━	━	━	━	━	━	
Ascertainment of multiplex families (FHQ, RFQ)			━	━	━	━	━	━	━	━	━	━	
Biospecimens collection from consenting participants				━	━	━	━	━	━	━	━	━	
Set up biospecimens bank				━	━	━	━	━	━	━	━	━	
Recruitment first degree relatives of multiplex families					━	━	━	━	━	━	━	━	
Data keying, checking, and analysis of FHQ and RFQ				━	━	━	━	━	━	━	━	━	

During the second grant year of the component project, we worked very hard to recruit every UC patients we could found. Until now, we interview 730 consenting participants from Taiwan university Hospital ,Chi-Mei hospital. and Chia-Yi Christian Hospital. We also collected blood and urine from consenting participants if they agree us to do it. First degree relatives of twenty-one multiplex families have been recruitment (family history prevalence of UC =2.9%), which implied that we have good opportunities to examine the effects of possible candidate genetic marker(s) and their synergistic interactions with environmental factors on UC. However, based on our limited personnel, it would be very difficult to recruit 150 multiplex families during grant period. In order to speed recruitment of multiplex families, we need more research assistants to recruit at least 60 multiplex families each year in the following two grant year. The detail arrangement of personnel in this project and related modified budget will be further described in the section B3. I hope the modified budget could be funded. Then, we feel confident of accomplishing the component study.

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