

EXTENDED ABSTRACTS

Predicting Individual Radiation Sensitivity: Current and Evolving Technologies

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In 2005, the National Institute of Allergy and Infectious Diseases (NIAID) of the U.S. National Institutes of Health established a Radiation/Nuclear Program to develop radioprotectors, mitigators and therapeutic agents to facilitate an effective medical response against radiological and nuclear threats. This program also supports development of biomarker/biodosimetry techniques and devices for rapid triage and treatment of radiation-exposed individuals after any radiological event.

In support of this program, NIAID in collaboration with the Columbia University Medical Center organized a workshop entitled “Predicting Individual Radiation Sensitivity: Current and Evolving Technologies.” The meeting was held at Columbia University in New York on March 17–18, 2008, and was attended by 86 participants from eight countries.

The background to the Workshop relates to the need for mass biodosimetry and mitigation/therapy after a large-scale radiological event, both in regard to short-term sequelae and also in terms of long-term end points such as carcinogenesis and heart disease. While retrospective dose estimates provide information about average risks, it is known from studies of higher-dose radiotherapy that there is considerable person-to-person variability in response to a given radiation dose. Thus the goals of this Workshop were to assess the significance of interindividual radiation sensitivity in terms of the aftermath of a large-scale radiological event and to assess whether the approaches used at clinical doses can be translated to lower doses or whether different approaches will be needed.

Session themes were:

- Individual Radiation Sensitivity in the Context of Radiological Emergencies
- Candidate Genes for Radiosensitivity
- Genome-wide Approaches
- Bioinformatics
- Future Developments

Radiation Countermeasures: The Need for Predictive Biomarkers

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National security experts have expressed increased concern in recent years about the threat of radiological and nuclear terrorism (1–3). Sce-

narios of concern include the surreptitious or overt dispersion of radioactive materials, attacks on nuclear power plants, and the detonation of stolen or improvised nuclear weapons. The latter scenario, while of low probability, poses the greatest challenge in terms of emergency preparedness and response because of the intense devastation and large number of injuries that such an attack would produce. Among the casualties of a nuclear detonation would be tens or hundreds of thousands of persons exposed to radioactive fallout downwind from the explosion. Such victims might be exposed to substantial doses of radiation but present (at least initially) without clear signs and symptoms of radiation toxicity or exposure. Similarly, persons exposed to radiation as a result of a radiological dispersion device might also present with minimal evidence of exposure. There is thus a need for rapid, accurate and sensitive diagnostic platforms that can confirm exposure and estimate the radiation dose absorbed by victims of radiation accidents or acts of terrorism. If mass

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radiation exposures occur, effective radiation biodosimetry will be required to optimize the allocation of scarce medical countermeasures and the delivery of supportive care.

Acute radiation syndrome (ARS) increases in severity as the absorbed dose of radiation increases and may involve multiple organ systems. Anecdotal evidence and clinical experience also suggest that interindividual differences in radiosensitivity may contribute to variability in the manifestations of ARS. Similarly, the delayed effects of acute radiation exposure on the lung, kidney, cardiovascular and other organ systems are dose-dependent in their expression but are also likely to be modified by individual and genetic factors. The elucidation of individual and genetic factors that influence clinical outcomes and the identification of sensitive and specific biomarkers of individual radiosensitivity or organ-specific injury will allow the further tailoring of therapy for victims of radiation accidents or acts of terrorism, particularly as new and more effective radiation medical countermeasures are developed. The benefits of identifying such factors and biomarkers could potentially extend to improved risk stratification for the stochastic effects of radiation exposure, including carcinogenesis.

The National Institute of Allergy and Infectious Diseases (NIAID) established the Radiation Countermeasures Research Program in 2005 to support the development of improved therapeutic and diagnostic countermeasures for radiation injury. NIAID funds research and development of medical countermeasures for ARS and the delayed effects of acute radiation as well as radionuclide exposures and is supporting the development of improved diagnostic platforms through its Centers for Medical Countermeasures against Radiation and in collaboration with the Armed Forces Radiobiology Research Institute. NIAID works in partnership with the Centers for Disease Control and Prevention, the U.S. Food and Drug Administration, and the Office of the Assistant Secretary for Preparedness and Response within the Department of Health and Human Services to develop, license, procure and deploy effective medical countermeasures against radiation threats.

With respect to medical diagnostics, NIAID envisions the establishment of an architecture to support immediate triage, dose estimation and radiation injury risk assessment. The technical requirements of such an architecture are that it have the capability for rapid screening of large populations, that it be sufficiently flexible to address different needs as these arise, depending on the scale of an event and extent of the injuries encountered, and that the tools employed be sufficiently accurate to guide clinical decision-making and improve population outcomes. The development of such an architecture will have medical or operational impact to the extent that it optimizes resource allocation, identifies patients requiring urgent medical assessment, reassures individuals concerned about their exposure, and improves risk assessment for the delayed or late effects of radiation exposure.

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The Radiation Oncology-Nuclear Terrorism Perspective²

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The emerging era of “personalized medicine” represents an extraordinary change in how disease risk, prevention and treatment will be

viewed and approached. For example, the predisposition to developing a malignancy is predictable to a subset of individuals at risk for breast and ovarian cancer (BRCA1 and 2), leading to steps as dramatic as prophylactic organ removal and the use of “chemopreventive” agents. Because the latter must be given for a sustained period, they must be of sufficiently low toxicity to ensure compliance and a satisfactory risk:benefit profile. As another example, the molecular signature of a tumor maybe used to estimate prognosis and to select therapy, as with gene expression arrays for breast cancer.

Predicting normal tissue toxicity is also being done at the molecular level. Polymorphisms in drug metabolizing enzymes are useful in predicting toxicity from chemotherapeutic agents and from anticoagulation (1). In a similar manner, certain enzymes have potential utility for predicting normal tissue toxicity, with the major DNA repair defects being examples. It is logical that some of the polymorphisms or mutations that lead to cancer susceptibility might lead to radiation sensitivity and not only increase therapeutic response but also increase the risk of normal tissue injury. Other polymorphisms in tissue and inflammatory response are being studied. Those that predict sensitivity would be useful in managing both radiation oncology patients and victims of a radiation terrorist event, particularly an improvised nuclear device that might produce a large whole-body or extensive partial-body dose with resultant acute radiation syndrome.

The utility of this knowledge depends on both the frequency of the underlying complication and the hazard ratio. Recent (2008) publications for predicting various cancer outcomes from SNPs for prostate cancer (2) had an HR of 1.5–~4, if four or five of five SNPs were present; lung cancer (2), HR of 1.5–~4 per gene for mutation in five genes and up to 15 for a “doublet” of genes; and breast cancer showed an HR of 1.5–~4 for various gene chips with variable numbers of genes (3). Thus most of these hazard ratios are around 2–3 or so, which would be an important difference if the baseline event is relatively high (i.e., increasing a 1% risk to 3% is not likely worth the cost of the test as opposed to increasing a 10% risk to 30%).

The long-term consequence of radiation-induced cancer is a feared concern for oncology patients and those exposed accidentally or intentionally. While the increased risk even for a dose of 5 rem is relatively low for an individual (~0.3% lifetime), exposure to large populations will create a major stress on both those affected and the healthcare system. The ability to measure exposure using biodosimetry will allow appropriate risk assessment, counseling and mitigation including behavioral modification (smoking), screening and, if available, the use of a “chemopreventive” agent.

Issues to consider (presented at the conference) are

1. Distinguishing needs for
 - a. Clinical radiation therapy (high dose, organ tolerance)
 - b. Managing acute radiation syndrome (ARS) and delayed effect of acute radiation injury (moderate dose)
 - c. Surveillance for radiation-induced carcinogenesis (lower dose)
2. Populations at risk: risk level, target organ—is there a particular subset of people for a particular test?
 - a. External irradiation versus internal contamination
 - b. Normal tissue injury: lung (high dose)
 - c. Combined injury: trauma plus radiation
 - d. Carcinogenesis
3. How does one deal with so many uncertainties in an event (exposure, neutrons, heterogeneous dose, dose rate)? Does the biodosimetry test used (cytogenetics, molecular) include components of both exposure and individual susceptibility (making a separate test of risk less helpful)?
4. How good is the test? Does it identify a sufficiently large population or at least one for which knowing the data matters in terms of management? If the expected outcome is a very rare abnormality, is it worth analyzing many people to find one?
5. Are multiple assays needed? How does one validate both the assay and the impact on an intervention (diagnostic, therapeutic) based on the assay?

² The opinions are not those of NCI or HHS but of the author.

6. Financial considerations—cost of test; cost of establishing a bona fide laboratory test (CLIA, ISO, etc.)
7. What difference does it really make? Will the absence of risk reduce healthcare costs? What intervention can be done based on the positive test?

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Possible High-Throughput Screening Logistics: Integrating High-Throughput Radiation Biodosimetry with High-Throughput Assays for Radiation Sensitivity

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There is clear utility for ultra high-throughput biodosimetry in the aftermath of a radioactive dispersal device (RDD) or an improvised nuclear device (IND). Applications are both for prediction and treatment of acute radiation effects and also for long-term assessment of carcinogenesis. However, as well as individual dose, individual radiation sensitivity plays a key role in determining both the early and late sequelae of radiation exposure.

High-throughput biodosimetry is well established as a long-term need for radiation threat countermeasures, but less attention has been paid to high-throughput assays for radiation sensitivity, in large part because predictors of individual radiation sensitivity, the subject of this Workshop, have yet to be established.

How might a high-throughput assay of individual radiation sensitivity work in practice? Can it be integrated with a high-throughput biodosimetry approach? We will briefly describe two of the approaches that we have taken toward high-throughput biodosimetry and discuss how a radiation sensitivity assay might be integrated into such systems.

The first approach is based on gene expression changes in response to radiation. We have developed dose-dependent gene expression “fingerprints” of radiation exposure, where a change in the mRNA expression in some tens of genes can be used to estimate a recent radiation dose. We are developing an inexpensive easy-to-read cartridge device to measure these dose-dependent gene expression fingerprints from a finger stick of blood. Should a gene expression fingerprint of radiation sensitivity be established, it would be relatively simple to incorporate this into the cartridge.

In a second possible approach, we are well advanced in the development of the RABIT (Rapid Automated Biodosimetry Tool), which is a fully automated ultra high-throughput robotically based biodosimetry workstation. It is again based on a sample of blood from a finger stick and uses *in situ* assays in a multiwell plate platform for either micronuclei or γ -H2AX foci. In this case one would not be able to assay for a mechanistically based predictor of radiosensitivity, but a more functional approach becomes possible. In this approach, the sample is split into two: One half is assayed for the biodosimetric end point of choice, while the other is irradiated, again in a high-throughput platform, with a dose sig-

nificantly higher than those anticipated from the radiological event, after which this part of the sample is assayed in exactly the same way as the other part. The controlled-irradiated sample will yield a functional estimate of radiation sensitivity to augment the dose estimate from the other sample. Because the volume to be irradiated is very small ($\sim 30 \mu\text{l}$), a circular array of five $\sim 4 \text{ mCi}$ (148 MBq) $^{90}\text{Sr}/^{90}\text{Y}$ β -particle-emitting seeds could be used for irradiation.

Genetic Predisposition for the Development of Radiation-Associated Cancer

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Ionizing radiation is the only established environmental risk factor in the development of brain tumors. However, only about 1–5% of previously exposed individuals develop such a tumor. It seems plausible, therefore, that personal susceptibility conferred by low-penetrance genes could facilitate tumor formation after the initiation effect of radiation.

Among the earliest sources of evidence of the increased risk associated with exposure to ionizing radiation was data from the Israeli tinea capitis cohort, which comprised 10,834 individuals who had been irradiated as treatment for this disease in the 1950s, and two matched nonirradiated population and sibling control groups. Long-term follow-up of this cohort demonstrated a significantly increased risk of both malignant and benign head and neck tumors in the irradiated group, with a relative risk as high as 9.5 for meningioma after a mean dose of 15 Gy to the brain. As a result of these findings, a law was established in Israel in 1994 requiring compensation to people who had developed deleterious health outcomes caused by this treatment.

By combining the original tinea capitis cohort with study populations derived from the compensation files and the Israeli Cancer Registry, a unique study population was created that enabled the examination of interactions between radiation and genetic factors in tumor development by conducting four group (exposed and non-exposed cases and controls) nested case-control studies.

An analysis designed to examine the possible role of several candidate genes involved in DNA repair and cell cycle control in radiation-associated tumors identified two out of 12 SNPs (ki-ras, ERCC2) that were associated with meningioma risk and two additional markers (Cyclin D1, P16) that showed an inverse effect in irradiated cases compared with sporadic meningiomas.

The involvement of additional candidate genes that play a major role in mediating cell cycle arrest, apoptosis and response to DNA damage, such as repair of double-strand breaks (e.g. the AT and BRIP1 genes), in radiation-associated tumors is now being tested.

Although familial clustering of meningiomas is extremely rare (<1%), further examination of this study group led to the identification of 17 families (11%), in which two to four first-degree relatives developed radiation-associated tumors. The unique situation in which several family members were exposed to radiation while others were not created a natural experiment that allowed us to simultaneously assess the effect of exposure and inheritance shown by familial aggregation of the disease among irradiated individuals within the same family. These findings imply that the occurrence of the tumor after the exposure is not a random event and most probably has a genetic component. We are currently analyzing this series of families to identify new susceptibility genes for radiosensitivity, using high-density SNP arrays to localize potential loci through linkage disequilibrium.

Identification of individuals genetically susceptible to ionizing radiation may help define subpopulations that would benefit from counseling to decrease their exposure to radiation and who can be targeted for early cancer detection schemes. These findings also demonstrate the need to

reevaluate the existing radiation protection guidelines and call for a more individualized approach in determining risk by level of exposure.

The Inherited Basis for Human Radiosensitivity

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The concept of radiosensitivity is clearly defined each day in radiation therapy clinics by the 2–5% of patients who develop adverse reactions to conventional doses of radiation. However, little is understood about why this happens or how to predict it. Since this adverse reaction to radiation can also be demonstrated in the laboratory in propagated cell lines from patients with certain disorders, such as ataxia telangiectasia, much of this adversity is probably genetic; it is inherent to the individual (1). This short overview will trace these concepts and some of the related methods for defining radiosensitivity from the early descriptions of chromosomal instability syndromes to modern molecular genetics. What then becomes obvious is a new overarching *XCIND syndrome*: X-ray sensitivity cancer, immunodeficiency, neurological deficiency, and double-strand break repair deficiency (2).

After World War II, a preoccupation with the possibility that excessive radiation could cause cancer gave rise to observations of chromosomal abnormalities in disorders associated with high cancer risk. Quadriradial configurations were noted in cells from Bloom's syndrome; t7;14 translocations were reported in lymphocytes from ataxia telangiectasia patients; Fanconi anemia karyotypes revealed excessive numbers of chromatin breaks. These disorders were also categorized as familial cancer syndromes. Today, the genes for most of these disorders have been identified and certain generalizations have become apparent (1). For one, these genes all play important roles in how rapidly DNA breaks are processed (often within seconds of damage) and how the integrity of the double strands is maintained, not only after it has been damaged (such as by radiation) but, more commonly, during cell division—when the smallest DNA aberration can become a lethal lesion. DNA is also “sliced and diced” (i.e., lengthened, shortened, excised or re-ligated) during the maturation of lymphoid cells, during viral infection and invasion of the genome, and during maintenance of chromosomal ends (telomeres). Some estimates suggest that over 1000 genes may be involved in DNA repair and processing, and a defect in any one of these genes can lead to serious consequences, often including radiosensitivity, cancer and/or immunodeficiency.

The common denominator of cellular radiosensitivity appears to be the repair of double-strand breaks (with no known exceptions at this writing!). Three main pathways can be appreciated: (1) homologous repair, (2) non-homologous end joining, and (3) the Fanconi pathway (3–5). These are listed in order of their phylogenetic appearance, the Fanconi pathway being the most modern. Non-homologous end joining is error prone compared to homologous repair, but it is faster than homologous repair and requires much less prior information about the DNA region in need of repair. It is the major repair pathway of mammalian cells. These pathways also function at different times during the cell cycle. This understanding has been gleaned from dissecting the repair defects of patients who are radiosensitive, cancer-prone and immunodeficient (Table 1).

To estimate the frequency of radiosensitivity in the general population, one must first define what is meant by radiosensitivity. Does this include only those individuals with adverse reactions to radiation therapy? No, because that would exclude the portion of the population who are also at risk but have never been exposed to radiation therapy. Thus, if 2–5% of radiation therapy patients experience adverse reactions, radiosensitivity in the general population must be at least that frequent. Adverse reactions after chemotherapy most likely encompass some of the same genes as those involved in radiosensitivity since many chemotherapeutic agents are also DNA-damaging agents. In the laboratory, one can identify hy-

TABLE 1
XCIND Syndrome Disorders

Ataxia telangiectasia
Nijmegen breakage syndrome
Rad50 deficiency
Mre11 deficiency (ATLD)
Ligase IV deficiency
SCID-ADA deficiency
SCID-RA (Artemis deficiency)
SCID-Cernunnos (XLF)
SCIDd-DNA-PKcs (dog/horses)
X-linked agammaglobulinemia
Fanconi anemia? ^a

^a Six complementation groups of Fanconi anemia patients have been shown to be radiosensitive by colony formation cell survival assay (6). The “?” reflects the fact that many FA patients do not meet all of the criteria for inclusion in XCIND syndrome; namely, they do not show severe immunodeficiency, nor do FA-D2 patients, for example, manifest a clearly increased incidence of cancer (7). Last, the FANC proteins are targeted to chromatin rather than directly to DNA double-strand breaks.

persensitivity to ionizing radiation by measuring the potential of cell colonies or clones to recover from radiation damage, such as by using a colony survival assay (5). This will, of course, be limited to estimating the radiosensitivity of cells that can form colonies *in vitro*, such as fibroblasts or lymphoblasts, and may be somewhat misleading as to the tissue- or organ-specific sensitivities. On the other hand, colony survival assays at least allow a comparison of radiosensitivity between individuals. Other assays, such as measuring postirradiation apoptosis or nuclear foci of DNA repair, can also be used; however, the gold standard is the colony formation cell survival assay.

Another approach to estimating the frequency of radiosensitivity in the general population is to use the carrier (heterozygote) frequency for each DNA repair or chromosomal instability disorder. This is seriously limited by the fact that while only about 16 have been defined to date (Table 1), there are most likely many others. Also, we cannot assume that in each disorder the heterozygotes will manifest the same degree of radiosensitivity. To determine this, better methods of detection will be necessary.

If one estimates, conservatively, that only 40 DNA repair disorders exist and that the carrier frequency is approximately 4/1000, then 160/1000 (16%) persons in the U.S. would be carriers of a DNA repair disorder. These individuals would be expected to tolerate lower radiation exposure levels than those of the general population. It also follows that if more sensitive laboratory methods could be developed for identifying these individuals, higher doses could be delivered to the remaining population with fewer adverse reactions and perhaps better efficacy of radiation therapy for cancer. This potentially “radiosensitive” population of one in seven persons should also be at a greater risk of radiation sickness in the event of a widespread nuclear exposure, and they would warrant more immediate medical intervention.

Thus the potentially radiosensitive population is comprised of (1) a small number of highly radiosensitive patients who are homozygous for DNA repair defects ($40 \times 1/100000 = 0.04\%$) and (2) a much larger but less radiosensitive population of heterozygotes or carriers ($40 \times 4/1000 = 16\%$). The latter may indeed be the same population among whom the 2–5% adverse reactions are noted. Further, one might expect that the incidence of cancer among such heterozygotes is also increased, as an extension of the increased cancer risk observed for ATM and NBS heterozygotes (8, 9). Therefore, this population should be overrepresented in radiation oncology clinics.

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Prediction of Normal Tissue Radiosensitivity from Polymorphisms in Candidate Genes

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The ability to predict normal tissue radiosensitivity prior to treatment has long been a goal in radiobiology. Over the last decade, increasing efforts have been made to establish associations between various genetic germline alterations and the risk of adverse reactions after radiotherapy.

It seems reasonable to assume that normal tissue radiosensitivity should be regarded as a complex trait that depends on the combined influence of several different genetic alterations. Single nucleotide polymorphisms (SNPs) make up 90% of naturally occurring sequence variation in the human genome, and SNPs in genes related to the biological response to ionizing radiation may affect clinical radiosensitivity. Rare genetic variants could also play an important role. It can be hypothesized that some genetic alterations inflict a specific impact on certain types of normal tissue damage, whereas others affect radiosensitivity in general. The genetic determinants of normal tissue radiosensitivity may also vary in terms of penetrance. Thus the “allelic architecture” underlying differences in normal tissue reactions may be rather complicated (1, 2).

So far, approximately 40 published studies have addressed possible associations between different genetic alterations in candidate genes and the risk of adverse normal tissue reactions after radiotherapy. Around half of these studies had a particular focus on single nucleotide polymorphisms. Though the studies have been relatively small and methodologically heterogeneous, preliminary indications have been provided that single nucleotide polymorphisms in the genes *TGFBI* and *ATM* may modulate risk of particularly late toxicity. In addition, rare *ATM* alterations may enhance complication susceptibility (2).

A meta analysis of nine published studies addressing the impact of the *TGFBI* position –509 SNP on the risk of late toxicity indicates that the position –509 TT genotype may constitute a marker of enhanced normal tissue radiosensitivity (OR 1.8, 95% CI 1.15–2.80). However, the distribution of the observations suggests that the result may be influenced by

publication bias. This finding emphasizes the need for large well-powered studies in the attempts to unravel the genetic basis of radiosensitivity.

In conclusion, we are still far from having an exhaustive understanding of the genetics that may underlie differences in the risk of normal tissue complication. However, recent technical advances and emerging insights into the structure of interindividual genetic variation open unprecedented opportunities to dissect the molecular and genetic basis of normal tissue radiosensitivity.

A first step would be to seek a confirmation of previously reported associations. Furthermore, novel high-throughput genotyping platforms enable broad-based candidate gene studies. Whole-genome association studies may represent an appealing alternative to the candidate gene approach (3). However, to fully exploit these new possibilities, well-planned large-scale clinical studies are mandatory. This calls for international collaboration.

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Heterozygous Animal Models of Radiation Sensitivity

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In the last several years, heterozygosity leading to haploinsufficiency for proteins involved in DNA repair pathways was shown to play a role in genomic instability and carcinogenesis after the induction of DNA damage. Since the effect of haploinsufficiency for one protein is relatively small, we hypothesize that defects in radiation response could be a result of the additive effect of heterozygosity for two or more genes critical for pathways that control DNA damage signaling, repair or apoptosis. To address this issue, mice or primary mouse cells haploinsufficient for one or two proteins, *ATM* and *RAD9*, related to the cellular response to DNA damage were examined for cell transformation, apoptosis and ocular cataracts. The results demonstrate that cells having low levels of both *ATM* and *RAD9* proteins are more sensitive to transformation by radiation, have different DNA double-strand break repair dynamics, and are less apoptotic compared to wild-type controls or those cells haploinsufficient for only one of these proteins. Also, mice haploinsufficient for both proteins develop ocular cataracts earlier than wild-type mice or mice haploinsufficient for only one of these proteins. Our conclusions are that under stress conditions the efficiency and capacity for DNA repair mediated by the *ATM/RAD9* cell signaling network depend on the abundance of both proteins and that in general, DNA repair network efficiencies are dependent on genotype and can vary within a specific range.

International Consortium to Create a Biorepository/Databank for the Performance of Genome-Wide SNP Association Studies

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The development of an assay capable of predicting which people exposed to ionizing radiation are most likely to manifest adverse radiation

effects in the form of tissue injury or development of a radiation-induced cancer represents a long sought-after goal. This is clearly important to potential victims of an act of radiological terrorism so that sensitive individuals receive particularly aggressive treatment for the radiation injuries they may have sustained. In addition, radiosensitive people will be after with particular care to enable early detection of a possible radiation-induced cancer. This is also of significance to radiotherapy patients to identify which people are most susceptible for complications after treatment. There have been numerous efforts to identify predictors of radiosensitivity. However, none of these assays have achieved an adequate level of sensitivity and specificity necessary for routine implementation. In recent years, attention has focused on the identification of genetic factors associated with radiosensitivity as the basis for an assay to predict which individuals are at increased risk for complications resulting from exposure to radiation.

A great deal of work has been performed to identify the candidate genes and single nucleotide polymorphisms (SNPs) in these genes that are associated with radiosensitivity. Although candidate gene studies have provided critical evidence supportive of a genetic basis for susceptibility for the development of radiation toxicity, this approach has reached an impasse in terms of its ability to provide findings that will translate into a useful predictive assay for the following reasons: (1) Although a number of studies have detected correlations between possession of a minor SNP allele with an increased incidence of either radiation injury or second malignancy, the results of early studies have not been validated routinely in subsequent work. (2) There is relative ignorance of the full spectrum of genes and proteins that are associated with the development of radiation injury and/or radiation-induced cancers. (3) Even if all of the important genes that encode the essential protein products associated with radiation toxicity were included in candidate gene studies, it is not certain that any of these genes would possess SNPs that would both alter protein function and be present at a high enough frequency in the population to be of importance. (4) Critical SNPs associated with radiosensitivity may not be located within genes, but in regulatory portions of the DNA.

Hence it is necessary for future research efforts to embark upon genome-wide studies to identify the SNPs associated with radiation toxicity to achieve the goal of creating a predictive assay for radiosensitivity. However, to perform genome-wide studies with statistical power to detect SNPs with modest relative risks associated with radiation toxicity, it is necessary to create a large biorepository and databank comprised of more than 10,000 well-characterized irradiated subjects. Therefore, an effort has been launched to establish an international consortium to create a biorepository/databank of people exposed to radiation that will be used to perform genome-wide studies to identify the SNPs predictive for the development of radiation injury and/or radiation-induced cancers. The subjects for these studies will be patients who received cancer radiotherapy. This is an ideal population for such a biorepository due to the large number of subjects, the well-defined dosimetry associated with their treatment, and the careful follow-up over a period of many years. Of equal importance is that these individuals received not only high radiation doses to the primary site of the cancer being treated but also moderate doses to adjacent normal tissues and scattered low doses to body sites distant from the main radiation field. Hence the use of radiotherapy patients can provide a basis for identification of SNPs associated with a susceptibility for the development of tissue damage and radiation-induced cancers resulting from exposure over a large range of radiation doses.

Candidate and Whole-Genome SNP Association Studies of Late Radiation Toxicity in Prostate Cancer Patients

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Background

Conformal radiotherapy is a common treatment option for patients di-

agnosed with prostate cancer. Despite advances in image-guided radiotherapy techniques, the bladder and rectum are the most common organs at risk for radiation exposure. A number of patients experience late tissue toxicity (>90 days after radiation therapy) resulting in bleeding from the rectum and/or bladder and other symptoms that represent adverse reactions to radiotherapy. Late tissue toxicity shows considerable variations in severity of symptoms between patients, possibly due to interindividual variations at the genomic level. The underlying premise for our work has been to (1) investigate single nucleotide polymorphisms (SNPs) for their association with the observed low or high normal tissue toxicity in a polygenic disease-phenotype model and, (2) at the whole-genome level, to describe novel potential markers potentially predictive of late tissue toxicity to undergo later validation.

Methods

We selected 82 cases of confirmed prostate cancer patients who underwent radiotherapy at the Cross Cancer Institute, Edmonton, Canada. Toxicity scores (RTOG scale) were documented for this cohort with 2–12 years of follow-up. Blood samples were collected after obtaining consent from patients and local IRB approval. DNA was isolated from buffy coat cells for genotyping assays. For the candidate approach we selected 49 SNPs in the *BRCA1*, *BRCA2*, *ESR1*, *XRCC1*, *XRCC2*, *XRCC3*, *NBN*, *RAD51*, *RAD52*, *LIG4*, *ATM*, *BCL2*, *TGFBI*, *MSH6*, *ERCC2*, *XPF*, *NR3C1*, *CYP1A1*, *CYP2C19*, *CYP3A5*, *CYP2D6*, *CYP11B2* and *CYP17A1* genes using the Pyrosequencing® technique. For the whole-genome studies, we used the Affymetrix platform for genotyping 262,000 SNPs that were arrayed on a single chip (NspI). Genotyping assays were carried out using the methods described by the manufacturer. SNPs that were non-polymorphic, with minor allele frequencies less than 5% and genotype distributions that showed deviation from the Hardy-Weinberg equilibrium test were discarded prior to analysis. The analysis program Haploview was used to analyze the remaining SNPs (~160,000) to detect linkage disequilibrium (LD) blocks, infer haplotypes, and tests of association for statistically significant differences between low (54 cases) and high (28 cases) toxicity groups for single locus and haplotypes.

Results and Conclusions

For the candidate approach, significant univariate associations with late rectal or bladder toxicity (grade 2+) were found for *XRCC3* (A>G 5' UTR NT 4541), *LIG4* (T>C Asp568Asp), *MLH1* (C>T, Val219Ile), *CYP2D6*4* (G>A splicing defect), mean rectal and bladder dose, dose to 30% of rectum or bladder, and age <60 years. On Cox multivariate analysis, significant associations with toxicity found for *LIG4* (T>C, Asp568Asp), *ERCC2* (G>A, Asp711Asp), *CYP2D6*4* (G>A, splicing defect), mean bladder dose >60 Gy, and dose to 30% of rectal volume >75 Gy (1).

In an exploratory whole-genome analysis, we detected statistically significant differences between low and high toxicity groups in haplotype associations from chromosomes 2, 4–8 and 12–21. Forty-four LD blocks were significant at $P < 10^{-4}$ and one block at $P < 10^{-5}$ after correcting for multiple marker testing (markers present in the LD block were used for correction and not the entire set of markers in the array). Statistically significant differences were also observed for the single locus association test.

Polymorphisms (in gene only or flanking regions) identified in whole-genome study with nominal values of $P < 0.05$ to 10^{-5} (without genomic correction for multiple markers) showed association with radiation-induced tissue response. Markers from cytoskeletal, proteases, inflammation, steroid metabolism and immune modulation (or those in proximity to these genes) along with several uncharacterized genes were present among the putative pathways identified in this study.

A validation cohort is planned to confirm the above observations with a larger sample size to identify novel genomic markers that are significant after Bonferroni correction. Collaboration between centers and formation

of centralized data and bio-sample repositories may facilitate studies with larger cohorts.

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Studies of Single Nucleotide Polymorphisms within the U.S. Radiologic Technologist Cohort

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In 1982, the U.S. National Cancer Institute, in collaboration with the University of Minnesota and the American Registry of Radiologic Technologists, initiated a study of cancer incidence and mortality among 146,022 (106,953 female) U.S. radiologic technologists (USRT) who were certified for at least 2 years between 1926 and 1982 (1). The cohort members are predominantly white (95%), and their current mean age is 58 years. During 1983–1989, 1994–1998 and 2003–2005, surveys were conducted that included detailed questions related to work history as a radiologic technologist, family history of cancer, reproductive history, height, weight, other cancer risk factors (such as alcohol and tobacco use), and information regarding a variety of health outcomes, including breast and thyroid cancer. Efforts to provide the best individualized organ radiation dose estimates are ongoing, but interim occupational radiation doses are based on badge dose probability density functions derived from monitoring badge readings (when available), historical dose data, and extensive individual work history data and applying dose conversion factors to estimate organ doses (including breast, thyroid and other organ sites) (2). Cumulative personal medical radiation exposure has been assessed two ways: first, by estimates based on the number of X-ray procedures the technologists underwent as patients and converted to a dose “score” with an equivalency in mGy (3, 4), and the second based on the summed number of procedures. Both occupational radiation doses to the red bone marrow and radiation exposure “score” from personal medical X-ray examinations, also to the red bone marrow, have been corroborated by FISH biodosimetry (3).

Biological specimen collections for nested studies of prevalent breast and papillary thyroid cancer were begun in 1999. Blood samples were collected from self-reported cancer cases and controls who were free of the cancer of interest at a similar time. Controls were frequency matched to cases based on year of birth in 5-year strata. Several USRT genetic studies of SNPs have been conducted for breast (859 cases, 1,083 controls) and thyroid cancer (167 cases, 491 controls).

For thyroid cancer, the genetic analysis has been limited to genotype main effects because the sample size is too small to assess meaningful interaction with radiation exposure and because the technologists were first exposed at around age 18, after which the thyroid gland is relatively insensitive to radiation. We observed a suggestive association with a SNP in the *RET* gene (rs1799939) that was strongest among women diagnosed under age 38 years (5).

Analyses of breast cancer have included approximately 165 different SNPs, with the aim of detecting interaction with ionizing radiation exposures from occupational sources and from undergoing diagnostic medical procedures as patients. Selected candidate variants were chosen from among DNA repair, apoptosis, inflammatory, oxidative and estrogen metabolizing genes and from among candidates identified in genome-wide association studies. Several SNPs have shown suggestive evidence of

radiation interaction (4, 6), including singletons in the genes *WRN*, *PRKDC*, *BRCA1*, *IL1A*, *ERCC2*, *PTGS*, *CYP1B1* and *H19*. However, some caution in interpretation is advised due to the numbers of comparisons conducted. The SNP in *H19* was analyzed because it was suggestively associated with breast cancer in a genome-wide study (7). Interestingly, the specific function of the *H19* gene is not known, but it is a maternally expressed imprinted non-coding mRNA tightly involved in regulating *IGF2*. It is difficult to discern whether *H19* might exert its influence directly through *IGF2* or indirectly by epigenetic phenomenon, since whole-body irradiation of BALB/c mice has been associated with an altered *H19* methylation pattern (8).

In summary, using candidate SNP approaches, we have found several variants that may alter risk of breast cancer in relation to radiation exposure. Replication of these findings is required but will be problematic because there are few, if any, other studies with both well-characterized radiation exposures in the low- to moderate-dose range and genetic material in sufficient sample sizes for meaningful comparisons.

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SNPs and Late Fibrosis

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Cancer patients receiving radiotherapy alone or in combination with chemotherapy display a large patient-to-patient variability in their risk of

developing normal tissue reactions. Although part of this variability can be ascribed to differences in treatment and patient characteristics, it has become increasingly clear that there might be an important genetic component. The term “radiogenomics” has been applied to the study of these genetic variants that are associated with the observed interpatient variability. One aim of radiogenomics studies is to develop tools for biologically individualized radiotherapy taking into consideration the individual risk of developing radiation-induced morbidity. Another aim is to improve the ability to reduce morbidity, through prevention or intervention, by increasing our understanding of the biological mechanisms behind.

Single nucleotide polymorphisms (SNPs) associated with increased risk of radiotherapy-induced late toxicity can be identified by different strategies, candidate gene association studies and genome-wide association studies. So far, association studies on genetic variants and radiation-induced morbidity have all been using the candidate gene approach. Based on mechanistic understanding of the radiation pathogenesis of early and late morbidity, a number of genes have been selected for further analysis. These analysis either have been restricted to known polymorphisms (most often SNPs but in a few cases also microsatellites) or used various resequencing approaches to identify rare variants. Results from these studies can be difficult to interpret and compare. First, radiation-induced morbidity is considered a quantitative complex trait, or phenotype, that can display continuous variation (and sometimes a threshold must be crossed before the phenotype is expressed). The genetic basis of quantitative complex traits often involves the effect of several genes. Some genes might affect the phenotype in an almost qualitative “all-or-none” way, but usually each causal gene makes only a small contribution to overall susceptibility, making it very difficult to identify the relevant genes. Furthermore, quantitative complex traits are often under environmental influences. Thus radiation-induced morbidity does not represent a single phenotype. Although patients affected with radiosensitive syndromes do seem to have a general increased risk of both early and late morbidity, there are clear indications that differences exist between the genetic component of various types of radiation-induced morbidity in unselected patients. Second, some studies have chosen to look at selected over-reactors, other studies include cohorts of consecutive patients, and some include reactors and non-reactors in a case-control-like manner. Any genetic associations identified in one of these types of studies may not necessarily be the same as in patients with significantly less or more severe phenotypes. Particularly for late effects, the length of follow-up is also very critical. Finally, possible differences in confounding factors is another reason that studies published so far are difficult to compare. Briefly, not all studies pay equal attention to the potential confounding effects of e.g. differences in radiation dose and type, target volume, target dose specification (especially when at a variable depth like tumor location), overall treatment time, fractionation, concomitant chemotherapy, juxtaposed skin surfaces, immobilizing and dose-modifying devices, and comorbidity. An example of the latter is connective tissue diseases that are associated with increased risk of late radiation morbidity and presumably have their own distinct genetic components.

An alternative to the candidate gene approach is genome-wide association studies. Genetic (SNP) association studies can be divided into different components. The first step is the identification of candidate association SNPs followed by validation of these SNPs. The next steps involve identification of the functional genetic variants linked with these SNPs and elucidation of the possible interactions between the genetic variants and any environmental factors. Successful genome-wide association studies in other research areas have been performed on the first two steps, identification and validation. One characteristic of these studies is that the phenotype investigated is disease occurrence that, compared to radiation-induced morbidity, makes it less complicated to distinguish cases from controls. Another characteristic of these studies is that they are performed by very large consortia. The current speed of development in genotyping technology and bioinformatics makes it difficult to provide detailed suggestions for how to perform genome-wide association studies in the field of SNPs and radiation-induced morbidity. Considering the complexity of the problem and the number of cases and controls needed,

formation of a large research consortium would be a large step in the right direction.

Meanwhile, a basic recommendation for future association studies on candidate genes is that data on the haplotypes of each gene (or different parts of the gene) should always be included along with data on the individual SNPs. Together with a number of other important points to consider, this recommendation can be found in the comprehensive guidelines by the NCI-NHGRI Working Group on Replication in Association Studies. Furthermore, candidate gene studies can still provide valuable information on the composition of quantitative complex traits and the functional consequences of different SNPs. One approach could be to break down the clinical phenotype into several intermediate phenotypes, also known as endophenotypes, that correlate with the clinical phenotype but are associated more closely with the genetic variants and may be portrayed by gene/protein expression patterns and may be applicable to pathway analysis. Examples of proposed intermediate phenotypes, which either have shown or may be expected to show some correlations with clinical end points, could include clonogenic survival, chromosome aberrations, mRNA expression patterns, differentiation, microRNA expression patterns, DNA repair, ROS scavenging, extracellular matrix remodeling, vascular damage, cytokine secretion, and cell-cell interactions. As an example, we recently presented data showing differential gene expression between patients with either low or high risk of radiation-induced fibrosis using patient-derived fibroblasts after irradiation. Another approach to identify human candidate genes and to study complex traits of radiation-induced morbidity is the use of mouse genetics.

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Predicting Radiotherapy-Induced Late Toxicity by Gene Expression Profiling

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Radiation is a common and effective anti-cancer therapy, but it leads to severe late radiation toxicity in 5–10% of treated patients. A pretreatment assessment of a patient’s probability to develop late toxicity could lead to increased cure rates and prevention of unnecessary suffering. Assuming that genetic susceptibility affects the risk to develop late effects, the cellular response of normal tissue to X rays could potentially be used to discriminate between patients who will and who will not develop late radiation toxicity.

In a retrospective study (1), it was tested whether the cellular response to X rays could be used to discriminate between patients with severe late complications and patients without symptoms after irradiation. Prostate carcinoma patients without evidence of cancer 2 years after curative radiotherapy were recruited and blood samples were collected from 38 individuals. The 24-h radiation response of stimulated lymphocytes after 2 Gy X rays was analyzed by gene expression profiling. The radiation response included a multitude of gene expression changes where thousands of genes were significantly modulated after X-ray exposure. Based on the

radiation response, two-class prediction analysis was performed either on the expression of individual genes or, to augment the power of classification, on gene sets consisting of genes grouped together based on function or cellular co-localization. The response to X radiation was variable across individuals, and the expression of only the most significant radioresponsive genes was not predictive of radiation toxicity. The classifier based on the radiation response of separate genes correctly classified the majority of the patients. However, when the gene products of the classifying genes were visualized in a human protein-protein interaction network, the high connectivity of the sub-network led to the idea and development of a gene set-based classification method. The classifier based on affected gene sets improved correct classification to 86%, showing the potential gain in analyzing genome-wide data sets with sets of genes instead of separate genes. On the individual level, about half of the patients were classified with high certainty using gene sets. In an independent validation set of 12 patients, the gene set classifier correctly predicted the toxicity status of eight patients. Among the classifying features, cellular functions such as ubiquitination, apoptosis and stress signaling were over-represented in both genes and gene sets. Interestingly, the apoptotic response appeared more robust in cells from patients that had not developed toxicity.

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Multiple Genetic Variants Associated with Risk of Adverse Reactions after Radiotherapy in Cancer Patients: A Large-Scale Candidate Gene Approach

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Human health is largely determined by a complex interplay between genetic susceptibility, environmental factors, and aging. We have focused on genetic elements that respond to radiation. The clinical radiosensitivity of normal tissue is likely to be a complex trait that is dependent on the cumulative effect of many minor genetic determinants. We have searched for polymorphisms that are associated with the radiation sensitivity of normal tissue of cancer patients who have undergone radiotherapy.

To date, a total of 2,327 patients involving 748 breast cancer patients, 309 cervical cancer patients, and 624 prostate cancer patients have been enrolled. Normal tissue reactions until the third month after completion of the treatment were graded according to the NCI/CTC scoring system. Late effects on normal tissues were graded according to the RTOG/EORTC and LENT-SOMA scoring systems. The selection of the candidate genes for SNP typing was based on our previous comprehensive gene expression analyses of human cultured cell lines and mouse strains and on the literature. A total of 140 genes were chosen and more than 1,300 SNPs of these genes have been typed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry system. We have attempted to use mainly haplotype analysis to identify effective markers.

This strategy was used to search for haplotypes associated with risk of early adverse skin reactions after radiotherapy in breast cancer patients. Global haplotype analysis indicated that estimated haplotypes at six loci, *PTTG1*, *MAD2L2*, *REV3*, *LIG3*, *RAD9A* and *CD44*, were associated with risk of adverse skin reactions. Comparison of risk haplotype to the most frequent haplotype at each locus showed haplotype GGTT in *CD44* was associated with adverse skin reactions risk. Five haplotypes, CG in *MAD2L2*, GTTG in *PTTG1*, TCC and CCG in *RAD9A*, and GCT in *LIG3*,

were associated with reduced adverse risk of skin reactions. No significant risk haplotype was observed in *REV3L*.

Identification of functionally important polymorphisms in radiation response genes may determine individual differences in sensitivity to radiation exposure. We believe these studies may pave the way for individual-oriented radiotherapy, which should have an improved therapeutic impact. The project will also further research on the molecular mechanisms of radiation sensitivity in humans.

Extracting Meaningful Biomarkers from Gene Expression Data

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To date, a very large part of the analysis of gene expression data has centered on methods to determine whether single genes or groups of genes exhibit patterns of gene expression that are sufficiently differential to allow accurate predictors of phenotype to be constructed based on their behavior. Work in this area has involved the use of data-viewing methods (clustering, principal components, etc.), distributional analyses (various forms of linear discriminant analysis), and classification techniques. Little analysis has been carried out to determine how fitting these methods are for the types and amounts of data available. For example, does the fact that biological systems do not function in ways where all of the components are not acting either randomly or independently have any impact on analyses that have these expectations as their basis? How well do analyses that converge on the right answer when the number of samples is huge and the number of variables small work when these conditions are inverted? How is correlation to a process to be interpreted when it is expected that a particular variable may have the same value when it is involved in process A as in process B?

Systems Biology and Understanding Radiation Sensitivity of Astronauts

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Astronauts on long-duration space missions face significant radiation risks from the protons and heavy nuclei that comprise the galactic cosmic rays and solar particle events. The principal risks of concern are carcinogenesis, acute radiation syndromes (ARS), and degenerative diseases including cataracts, central nervous system effects and heart disease. The biological effects of high-linear energy transfer (LET) radiation in space such as heavy nuclei and secondary neutrons have been shown to be different in both quantitative and qualitative ways from terrestrial forms of low-LET radiation, leading to large uncertainties in risk estimates. Because of the high energies of cosmic rays, radiation shielding is ineffective and organ exposures in the range of 0.75 to 1.5 Sv are expected for a Mars mission. Identifying individuals with resistance to radiation could benefit space exploration by reducing the high costs to launch shielding into space and ensuring that risk acceptance levels are not exceeded.

Space exploration benefits from research studies that will provide the basic understanding of individual sensitivity aimed at patients treated with radiation or accident victims from a radiation terrorism event. However, there are differences in the mechanisms of biological damage and response pathways between low-LET and high-LET radiation that must be considered. Studies have shown differences in gene expression patterns and post-transcriptionally modified protein levels between X rays and simulated space radiation using beams generated at particle accelerators. Genetic and epigenetic factors that play a role in determining radiation

sensitivity and resistance for astronauts will need to consider cancer as well as ARS and degenerative risks for long-duration space missions. The possibility of multiple disease types increases the need for systems biology approaches that can integrate information on potential high- and low-penetrance genes, including the role of SNPs in radiation response. The population of astronauts of about 120 is a highly selected group of healthy individuals. Astronauts are often employed by NASA for as long

as 20 years, and they may participate in several space missions. This provides an opportunity to consider potential interactions between genetic factors in individual sensitivity with biological factors related to aging as well as radiation exposure. Systems biology methods can be pursued to integrate information from biomarker data from multiyear samples including obtaining pre-flight calibration data from samples exposed to radiation and that follow post-flight outcomes.