Incorporating genetic biomarkers into predictive models of normal tissue toxicity

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Abstract

There is considerable variation in the level of toxicity patients experience for a given dose of radiotherapy that is associated with differences in underlying individual normal tissue radiosensitivity. A number of syndromes have a large effect on clinical radiosensitivity, but these are rare. Amongst non-syndromic patients, variation is less extreme, but equivalent to a ±20% variation in dose. Thus, if individual normal tissue radiosensitivity could be measured, it should be possible to optimise schedules for individual patients. Early investigations of *in vitro* cellular radiosensitivity supported a link with tissue response, but individual studies were equivocal. A lymphocyte apoptosis assay has potential, and is currently under prospective validation. The investigation of underlying genetic variation also has potential. Although early candidate gene studies were inconclusive, more recent genome wide association studies (GWAS) are revealing definite associations between genotype and toxicity and highlighting the potential for future genetic testing. Genetic testing and individualised dose prescriptions could reduce toxicity in radiosensitive patients, and permit isotoxic dose escalation to increase local control in radioresistant individuals. The approach could improve outcomes for half of the patients requiring radical radiotherapy. As a number of patient- and treatment-related factors also affect risk of toxicity for a given dose, genetic testing data will need to be incorporated into models that combine patient, treatment and genetic data.

Keywords: GWAS, SNP, normal tissue, radiosensitivity, radiotherapy

Introduction

Radiotherapy (RT) is one of the most effective treatments for cancer [1]. It is needed in the care of ~50% of cancer patients at some time in their illness. As the lifetime risk of cancer for people born since 1960 is estimated to be >50% [2], RT will ultimately be required for a quarter of the population. It forms a major part of the treatment plan for 40% of those who are cured and is primarily responsible for cure in 16%. Around 60% of patients undergoing RT are treated with curative intent [3]. The incidence of cancer in the UK is ~331,000 cases per annum [4], so that radical RT is used in around 100,000 patients each year.

The success of RT in eradicating a tumour depends especially on radiation dose, which is limited by the tolerance of surrounding normal tissues. Patients treated to the same curative dose vary in the toxicity they experience. A minority have no observable effect, most have clinically mild or moderate changes, and a few suffer serious normal tissue complications that may even be life-threatening. The incidence and severity of normal tissue damage is radiation dose dependent. However, even mild or moderate damage can have a substantial negative impact on patient-reported quality of life, and requires consideration. Selection of the appropriate RT is based on a balance between lowering the dose to keep the incidence of severe normal tissue complications at an acceptably low level, and raising the dose to increase the probability of local control. However, at present, this is done only on a population level, without the possibility of personalisation based on individual normal tissue tolerance.

Toxicity can be reduced by using advanced RT techniques which limit normal tissue doses, especially intensity modulated radiotherapy (IMRT) [5-13], addressing physical individualisation. All modern RT includes a substantial component of physical individualisation, which is not yet matched by the biological equivalent. Developments in RT, including the ability to combine physical and biological individualisation, will make an essential contribution to the Cancer Research UK vision of curing 75% of cancer patients in 20 years' time [14]. This article addresses the issue of biological individualisation of RT, which is a goal which should be reached well within this time frame, offering better cure rates with less toxicity for patients with cancer.

Background

The first descriptions of individual variation in toxicity

The first documented illustration of variation in toxicity following RT was reported by Holthusen in 1936 [15]. The evidence for individual variation in radiosensitivity led to the development of studies aimed at measuring radiosensitivity to predict a cancer patient's risk of toxicity. The variation was hypothesised to have a genetic basis, even though these efforts pre-dated the development of the necessary genotyping technology to prove this. Initially laboratory measurements of radiosensitivity were developed to attempt to predict normal tissue toxicity. The earliest studies focused on individuals with very severe toxicity, many with heritable syndromes, including ataxia telangiectasia (AT). Fibroblasts cultured from skin samples of such patients were shown to be unusually radiosensitive using clonogenic assays [16-21]. Clonogenic assays assess reproductive integrity, i.e., ability of single cells to form a colony with a minimum of 50 cells (representing at least 5-6 cell divisions) [22]. These studies showed a wide range of sensitivity, largely because of the inclusion of cells from patients with genetic syndromes typically associated with DNA damage recognition and repair defects, causing severe clinical and cellular radiosensitivity,

Efforts to develop predictive testing based on cellular radiosensitivity

With the demonstration in the 1980s that there was variation in fibroblast radiosensitivity between cells cultured from individuals both with and without known genetic syndromes [17, 23-25], studies were set up to investigate the relationship between cellular and clinical radiosensitivity with a goal of developing a test to predict a patient's likely reaction to RT. The first studies were retrospective and compared patients who developed severe reactions to RT to those with no/minimal toxicity. Toxicity was typically relative, with some patients probably not expressing really severe reactions, which presented a problem of discrimination in the clonogenic assay. The results suggested some value in cellular sensitivity testing, but without providing clear proof of a link between cellular and tissue radiosensitivity.

The next step saw several small studies of patients whose toxicity had been quantified more objectively. Each of these showed a correlation between cellular sensitivity and normal tissue response [26-29]. Although the results, individually and collectively, were encouraging, the relationship between cellular sensitivity and normal tissue response could

not be replicated in larger studies using the clonogenic assay with fibroblast cultured from skin samples [30]. Better results were obtained using lymphocytes [31].

Since deriving fibroblast lines from skin samples and performing the necessary clonogenic assays (in triplicate) takes 6-8 weeks and is labour-intensive, interest moved to investigating more rapid assays that would have greater clinical utility. The main ones studied have been expertly reviewed elsewhere [32], and include: chromosome damage assays, including include the 'micronucleus' and G2 lymphocyte assays; DNA damage, including the 'comet' assay; assessment of apoptosis; the ability of fibroblast to undergo radiation-induced differentiation; and alteration in telomere length. Combinations of assays have also been tested. Despite considerable effort, none of these methods proved reliable in a clinical setting. An important reason may be that the differences between cells from normal (as opposed to syndromic) patients are rather small, and of similar magnitude to the variability in the assays. Another important reason may be that the response of cultured cells might not be sufficiently comparable to the response of whole tissues, in which the microenvironment could play an important role in radiation-induced damage. Finally, the quality of dosimetry and reporting of clinical toxicity must be well controlled, but in general studies seeking to correlate sensitivity assays with clinical outcome have addressed these issues.

There is also interest in measuring the expression of cytokines in serum/plasma. A combined two centres' analysis of 165 patients with non-small cell lung cancer showed that elevation of and plasma transforming growth factor (TGF)-β1 during RT predicted for lung toxicity [33]. However, despite early reports of a correlation [33-35], not all studies have demonstrated a relationship [36], and more work is certainly required [37].

Studies have also attempted to derive gene expression signatures [38-41]. This work is challenging when attempting to measure radiosensitivity due to the need to choose whether to investigate baseline gene expression or radiation-induced expression, and has not yet been fruitful.

A single exception, the lymphocyte apoptosis assay, has emerged which appears to have potential [42]. Lymphocytes from patients with a variety of cancers who experienced severe late RT reactions, exhibited an impaired *in vitro* apoptotic response after 8Gy. For 348 patients evaluated at 2 years, or who developed a grade ≥2 toxicity before that, the area under

the receiver-operating characteristic curve was 0.83 (95% CI, 0.78-0.87), and the negative predictive value for grade 3 toxicity was 98.5%. At this level of prediction, it might be possible to undertake modest dose escalation. It is still unclear whether it can be used as a clinical predictive test, but it is currently being further evaluated [43].

Challenges with radiosensitivity assays

There are several problems associated with research aimed at testing whether laboratory measurement of radiosensitivity predict clinical radiosensitivity. First, assays are not standardised and there have been few attempts to ensure transferability across laboratories. Second, the studies involve different radiation doses, dose rates, and assay conditions. Third, patient cohorts are heterogeneous. Some studies involved severe atypical reactions, others investigated unselected patients. The factors determining radiosensitivity might differ between these two groups. Fourth, study designs vary considerably and few involve power calculations and multivariate analyses. Fifth, reproducibility is rarely reported but cell based assays tend to have a large experimental variability relative to inter-individual variability in radiosensitivity. Progress in the area requires standardised approaches for measuring radiosensitivity which are transferable across laboratories, and the establishment of guidelines for carrying out studies.

Challenges with clinical studies

A key challenge in clinical studies is to control for other factors which affect toxicity (Table 1). The main recognised determinants of radiotherapy toxicity relate to physics (radiation dose, dose rate, dosimetry, dose inhomogeneity, treatment volume), treatment (interaction with other modalities such as surgery, chemotherapy, hormone therapy, medication such as statins), patient factors (age, haemoglobin, smoking, co-morbid conditions such as diabetes & collagen vascular disease) and genetics [32]. Other factors which have been hypothesised but not confirmed to influence clinical radiosensitivity include sex, ethnicity, body mass index, diet, and alcohol consumption.

Genetic variation influences clinical radiosensitivity

Several clinical studies have shown that inter-patient variability in toxicity amongst non-syndromic patients receiving RT is greater than intra-patient variability and some have

suggested that, after controlling for other factors, the genetic component could be as high as 80% [44-46].

Heritability is defined as the proportion of phenotypic variance in a population attributable to additive genetic factors. Heritability of a disease is usually demonstrated by studies comparing phenotypic variance between twins or wider family members. However, evidence of heritability of RT toxicity is difficult to obtain due to the need for prospective toxicity data collection in cancer survivors and their close family. Thus, there is limited literature on the heritability of radiation toxicity. Scott et al. used a chromosome damage assay to investigate the radiosensitivity of first-degree relatives of 16 sensitive and eight 'normal' breast cancer survivors [47]. Sixty-two percent of first-degree relatives of sensitive patients were also radiosensitive compared with 7% first-degree relatives of 'normal' patients [48]. Unfortunately, this assay did not transfer well between laboratories, but recent studies of in vitro cellular radiosensitivity have suggested estimates of heritability of between 60% and 80% [48-53].

Syndromes associated with clinical radiosensitivity include AT [16], individuals with LIG4-syndrome [54], and individuals with Nijmegen Breakage Syndrome [55] involving *ATM*, *LIG4*, and *NBN* respectively. Such radiosensitivity syndromes illustrate that specific genes influence clinical radiosensitivity. These syndromes, characterised by Mendelian inheritance of germ-line mutations in genes involved in the detection of DNA damage or DNA repair (the DNA damage response, DDR), result in genomic instability and cancer pre-disposition. These syndromes are rare and probably of little relevance when assessing radiosensitivity in most cancer patients undergoing RT.

Radiogenomics

Candidate gene studies

Current understanding is that radiosensitivity is an inherited polygenic trait, dependent on the interaction of many genes/gene products involved in multiple cell processes [32, 56, 57]**.** One way to study common genetic variation is to use single nucleotide polymorphisms (SNPs). Most SNP association studies of RT toxicity, published to date, have used a candidate gene approach in which a set of SNPs are selected on the basis of a hypothesized effect on one or more genes whose protein products are involved in cellular pathways known to be involved

in radiation response. For example, radiation-induced cell killing, for which DNA damage is a major mechanism, is thought to be a triggering event in the development of RT toxicity. Additionally, the release of cytokines is considered to initiate biological responses in multiple cell types leading to the development of late toxicity. The focus of candidate gene studies has thus been on genes involved in DNA damage recognition and repair (e.g. *ATM*, *BRCA1*, *BRCA2* and *TP53*), free radical scavenging (e.g. *SOD2*) and anti-inflammatory response (e.g. *TGFB1*). Most studies, reviewed elsewhere [56, 58, 59], have been underpowered, including fewer than 500 samples. The studies tested many SNPs without adjusting for multiple comparisons and, although many reported positive associations, findings have proved difficult to replicate. One exception is a candidate gene study of 2036 women whose toxicity was scored after RT for breast cancer, which included a validation cohort to confirm the findings, and suggested a link between variation near the tumour necrosis factor alpha (*TNF-α*) gene and toxicity [60].

In contrast, a large independent validation study of the SNPs studied in candidate genes did not replicate previously reported late-toxicity associations, suggesting that the hypothesis that published SNPs individually exert a clinically significant effect could be excluded [61]. In addition, the international Radiogenomics Consortium collected and analysed individual patient level data from both published and unpublished studies of SNPs in *TGFB1*, encoding the pro-fibrotic cytokine TGF-β1. In this meta-analysis of 2782 patients from 11 cohorts, no statistically significant associations between either fibrosis or overall toxicity and rs1800469 genotype were observed [62].

These results overall demonstrate the difficulty of using small candidate gene studies, which presupposed that the biology of normal tissue toxicity is comprehensively understood. A different approach was needed, which was not subject to this limitation, namely genome wide association studies (GWAS).

Genome wide association studies (GWAS) in Radiogenomics

With the rapid reduction in cost of genotyping came the possibility of carrying out GWAS to identify new genes associated with toxicity, with no *a priori* assumptions about which genes might be important. The first published radiogenomics GWAS was a small pilot study among African American men treated with RT for prostate cancer. SNP rs2268363 within the *FSHR*

(follicle-stimulating hormone receptor) gene, involved in testes development and spermatogenesis) was associated with erectile dysfunction at a genome-wide level of statistical significance ($p=5.5 \times 10^{-8}$) [63].

Following this encouraging result, three larger radiogenomics GWAS have been undertaken and have uncovered additional risk loci at similarly appropriate levels of significance. In one, an association was found with late rectal bleeding following prostate cancer radiotherapy which approached genome-wide significance [64]. Of note, most of these early-identified SNP associations have been specific for toxicity in a particular tissue (for example rectal bleeding [64]). This suggestion that SNP associations can be specific to particular tissue types has been backed up by other studies.

A GWAS of late toxicity, incorporating both prostate and breast radiotherapy patients and using the STAT score of overall toxicity [65], identified a greater number of SNPs than expected by chance at a nominal significance level (Figs 1 & 2), although no individual SNP reached the accepted threshold for genome-wide significance [66]. This study provided important evidence that there are likely to be many SNPs truly associated with late radiotherapy effects that will be uncovered by adequately powered studies of increased sample size.

A third GWAS has identified a putative locus on chromosome 2q24.1, within the *TANC1* gene, associated with overall toxicity (a measure encompassing both urinary and rectal effects) following radiotherapy for prostate cancer (p-value 4.6×10^{-11} [67].

It is interesting that the putative loci reported so far are not close to obvious radiation-associated candidate genes, which is consistent with current opinion that we have insufficient understanding of the molecular pathogenesis of RT toxicity as a polygenic phenotype. Information from GWAS of other phenotypes suggest the allelic architecture underlying radiosensitivity will include a spectrum ranging from rare, highly penetrant to low-risk common alterations [68].

The increasing recognition that GWAS must include tens or even hundreds of thousands of patients [56, 57, 68] led to the establishment of a Radiogenomics Consortium (RGC) in 2009 [69]. The consortium created a vital link between existing collaborative groups [64, 66, 70-

73]. The consortium provides a route for: sharing expertise and quality assurance procedures; developing best practices for data collection; pooling data; and carrying out replication studies, which are so necessary given the small sample sizes available in the constituent studies of the consortium. The RGC recently published the STROGAR guidelines [74], aimed at improving the quality, transparency and completeness of radiogenomics research reporting. The Radiogenomics Consortium has also provided a platform for conducting a meta-analysis of four GWAS of late effects from prostate cancer radiotherapy, including the three aforementioned studies. This is the most statistically powerful radiogenomics GWAS to date, including over 1,600 men treated with radiotherapy for prostate cancer. Additional efforts are underway to perform GWAS meta-analyses among breast cancer and head and neck cancer radiotherapy patients.

Sample sizes available within the RGC remain small by comparison with other GWAS consortia. Consequently, not all the currently reported putative radiotoxicity associations are likely to be replicated. Moreover, the reported loci do not yet explain sufficient variability in toxicity to be useful for the long-term goal of predictive SNP profiling. For comparison, consortia formed to identify breast and prostate cancer risk loci (BCAC: http://apps.ccge.medschl.cam.ac.uk/consortia/bcac/; PRACTICAL: http://practical.ccge.medschl.cam.ac.uk) now have sample sizes in the order of 100,000 enabling them each to confirm almost 100 common genetic risk loci. The difference in risk of breast cancer between women at the very top and bottom of the SNP risk-profile developed from these loci is roughly 10 fold [75]. Sample sizes available within the RGC will continue to expand, allowing discovery and confirmation of further novel risk loci, and ultimately SNP risk-profiles for RT toxicity.

Many other challenges remain in radiogenomics that are related to heterogeneity in RT schedules between centres and countries; variable recording of RT toxicity; use of multiple toxicity scoring schemes; the multiple anatomical sites (e.g. breast, head and neck, prostate) and multiple toxicity endpoints associated with each site; the lack of consensus on the best time point for reporting toxicity; there being no standardisation of collection of additional risk factors. In addition there is a need to improve methods for incorporating dose data, including the actual dose received by surrounding normal tissues (accumulated dose or D_A: see below).

What probability should be considered statistically significant in a GWAS?

A specific issue for GWAS is the appropriate p-value considered to be statistically significant. The early literature reporting genetic association studies was littered with reports of associations that subsequently failed to replicate in independent studies. This may have been due to low frequencies of risk allele, non-causal correlations, and limited power in small studies. However, the major reason for the failure to replicate has been the use of inappropriately high p values, leading to false positives. Specifically, the conventional value $p < 0.05$ is an inappropriate threshold [32], with a p value $< 5 \times 10^{-8}$ accepted widely as denoting genome-wide significance.

Discussion

Using predictive tests of normal tissue sensitivity to alter treatment

The overriding motivation for predictive testing is to be able to tailor RT for an individual patient to improve outcome [76, 77]. At first sight, altering treatment to reduce toxicity in the 10% most sensitive patients would seem to be the priority. However, this applies to a minority of the population. An additional option is to also dose escalate more resistant patients, which, with a suitable division according to sensitivity, might account for 40% of patients [56, 78]. Together, these two groups could constitute half of the patients receiving RT.

Figure 3 shows a possible approach to the alteration of treatment based on a predictive test of individual radiosensitivity. This might be based on a conventional radiosensitivity assay or on a genetic 'signature', or possibly a combination of both. Clinical implementation would also need to take into account patient- and treatment-related factors as well as physical dose, which also alter an individual's risk of toxicity (Table 1). The figure shows the population of RT patients divided into 3 groups: the most sensitive 10%, the intermediate 50%, and the most resistant 40% [78]. This approach would allow potential alteration in management for half of the patients receiving RT, and has the attraction of aiming to identify patients at the extremes of the distribution of sensitivity.

In terms of altered management, hyperfractionation can reduce toxicity with no risk of loss of local control or to allow for dose escalation [79]. In an EORTC head and neck study [79], hyperfractionation allowed an additional 10.5 Gy to be given, over the 70 Gy standard arm, a

percentage difference of 15%. For suitable radiosensitive patients hyperfractionation would be an ideal choice. Suitability would depend on the hyperfractionation expanding the therapeutic window, which would be contingent on the α:β ratio of the tumour being greater than that for the normal tissue. For head and neck cancer clinical evidence demonstrates that this is the case [79]; for prostate cancer the question remains open, but should be largely resolved by results from the CHHiP trial [80].

For more resistant patients, an increase in dose should be possible. This could be designed to be isotoxic, that is delivering the same risk or level of toxicity for this subpopulation as for the general population (without predictive testing), or to be hypotoxic, avoiding most or all of the toxicities seen in the general population by choosing dose escalation to a slightly less high dose. This might also provide an opportunity to add novel targeted or existing cytotoxic agents.

Incorporating physical dose as a predictor of toxicity

The conventional approach to modelling toxicity risk involves evaluation of the RT dose plan, with some consideration also given to other treatment- and patient-related factors (Table 1) [81, 82]. Such predictive clinical models are effective, and could easily be extended to incorporate genotyping data.

A development of interest regarding use of the physical dose plan relates to the potential use of the total accumulated (delivered) dose (D_A), as distinct from the planned dose [83]. In principle, this can be done by using daily image guidance studies to identify the position of critical structures, and then re-computing the dose for each daily treatment. Summated, these provide an estimate of D_A. A small cohort study investigating rectal D_A in men receiving RT for prostate cancer has shown that the average position of the rectum is the same as the planning scan in most patients, but with substantial day-to-day variation [84]. This suggests that, at the population level, the RT plan is a good surrogate for D_A, but that for individual patients this may not be the case. In this small cohort, daily positional differences translated into differences in dose in all 10 cases studied, and were substantially higher than planned in one patient, and appreciably lower in six [85]. If the approach can be generalised, D_A could usefully be added into predictive models, and could also be useful in exposing genetic variation more effectively.

Conclusions

GWAS are at last revealing polymorphisms associated with toxicity risk, and more are likely to be identified in the near future [57, 66]. The possibility of a genetic predictive risk 'signature' is therefore promising. In addition, the biological hypothesis-free GWAS approach is likely to increase our understanding of the underlying biology of radiation normal tissue effects. The lymphocyte apoptosis assay is currently undergoing further evaluation [43], and if validated might provide an additional rapid predictive test to screen for either hypersensitive or relatively resistant patients.

Developments in technical RT will also contribute to improved understanding of dose-volume-response relationships, and through that to better normal tissue complication probability models. Ultimately, the aim will be to develop models of individual risk, combining treatment, patient and genetic factors to achieve optimal individualisation of treatment.

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Tables

Table 1. Key determinants of clinical radiosensitivity.

Physical dose factors	Treatment factors	Patient factors
Dose	Surgery	Age
Dose rate	Post-surgical cosmesis	Haemoglobin level
Dose homogeneity	Chemotherapy	Smoking
Treatment volume	Hormone treatment	Diabetes
	Statins	Collagen vascular disease
		Genetics

Figures

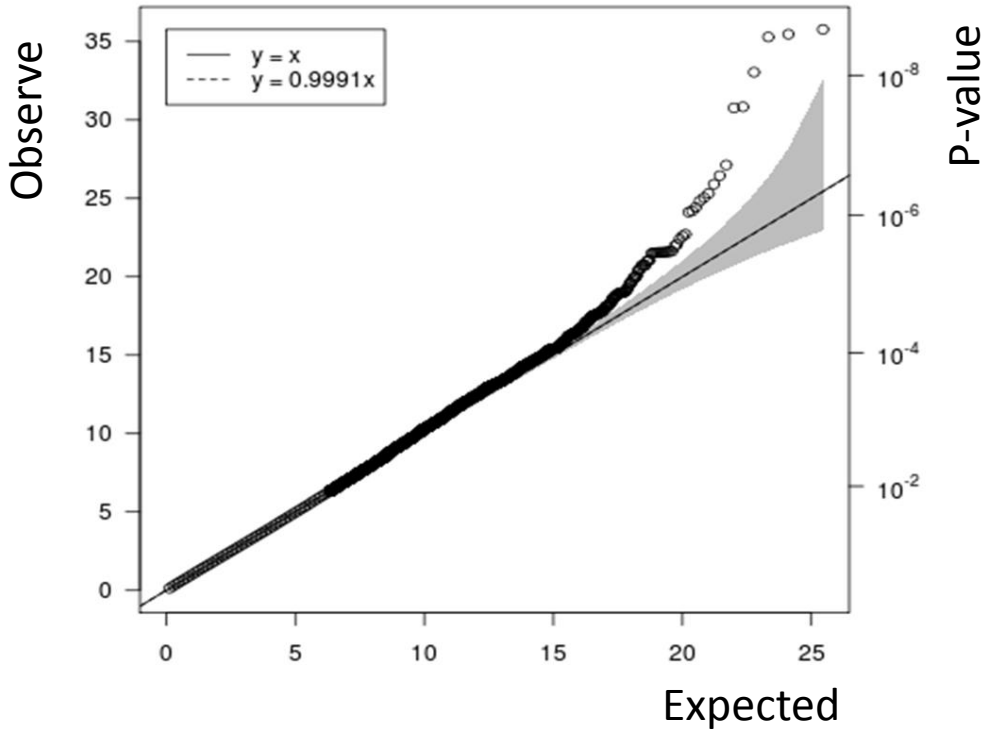

Figure 1. Example of a Q-Q plot, addressing the relationship between genetic variation and nocturnal frequency in prostate cancer patients. The Q-Q plot shows P values, specifically the observed P values versus those expected under the null hypothesis of no association between nocturnal frequency in a group of prostate patients and genotype in multivariate analysis (MVA).

In a GWAS study many hypotheses are simultaneously tested as each SNP is assessed for association with the outcome measure. It would be expected that there would be some low P-values obtained due to this multiple testing. A Q-Q plot is a visual means of assessing whether the observed P-values from a GWAs study deviate significantly from the P-values that would be expected from chance alone. If the distribution of p-values is as expected from chance then the points will lie along the line y=x. If the plot significantly deviates upwards away from the line y = x for the lowest 10% of P-values, as shown here, this is evidence of an association between common SNPs and the toxicity endpoint measured, even if the SNPs themselves are not identified.
(Image reproduced with permission from Radiotherapy & Oncology - requested).

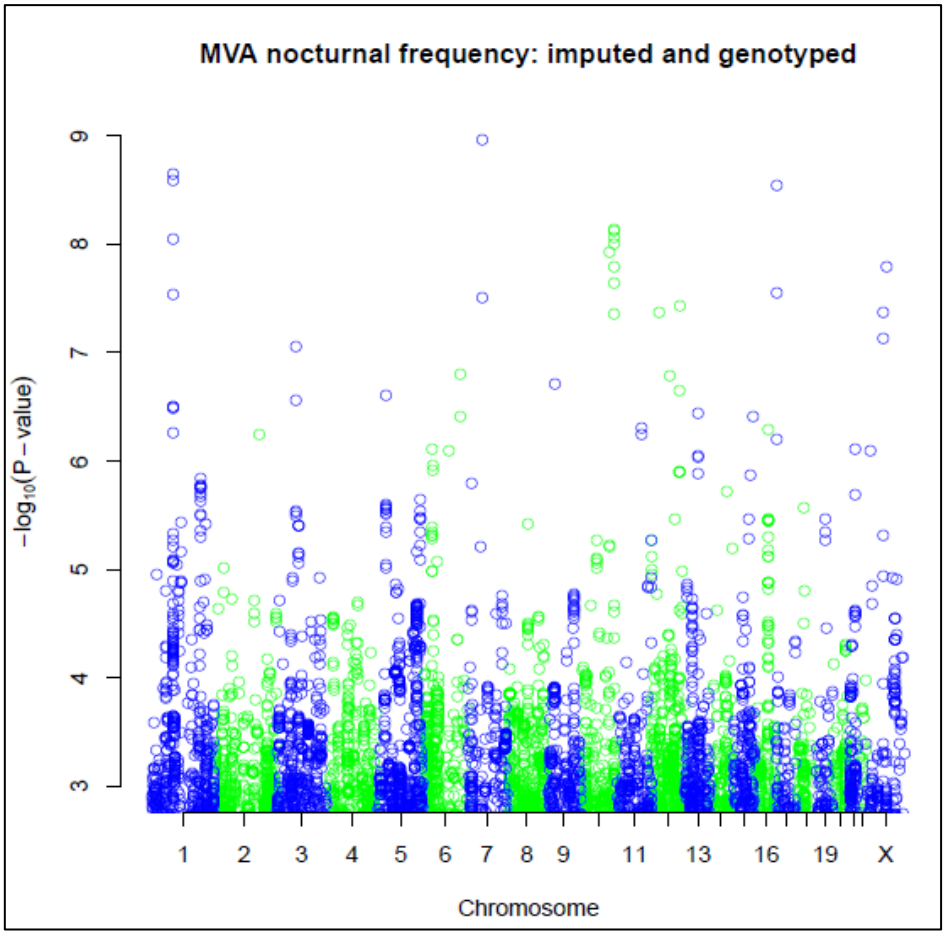

Figure 2. Example Manhattan plot from the first stage GWAS [66] showing observed log10P-values vs. SNP position from multivariate analysis of nocturnal frequency in men treated with radiotherapy for prostate cancer. A few chromosome regions contain SNPs which show evidence of association as shown by points representing P-values $< 5 \times 10^{-8}$. When the additional cohorts were included, none of the associations remained significant.

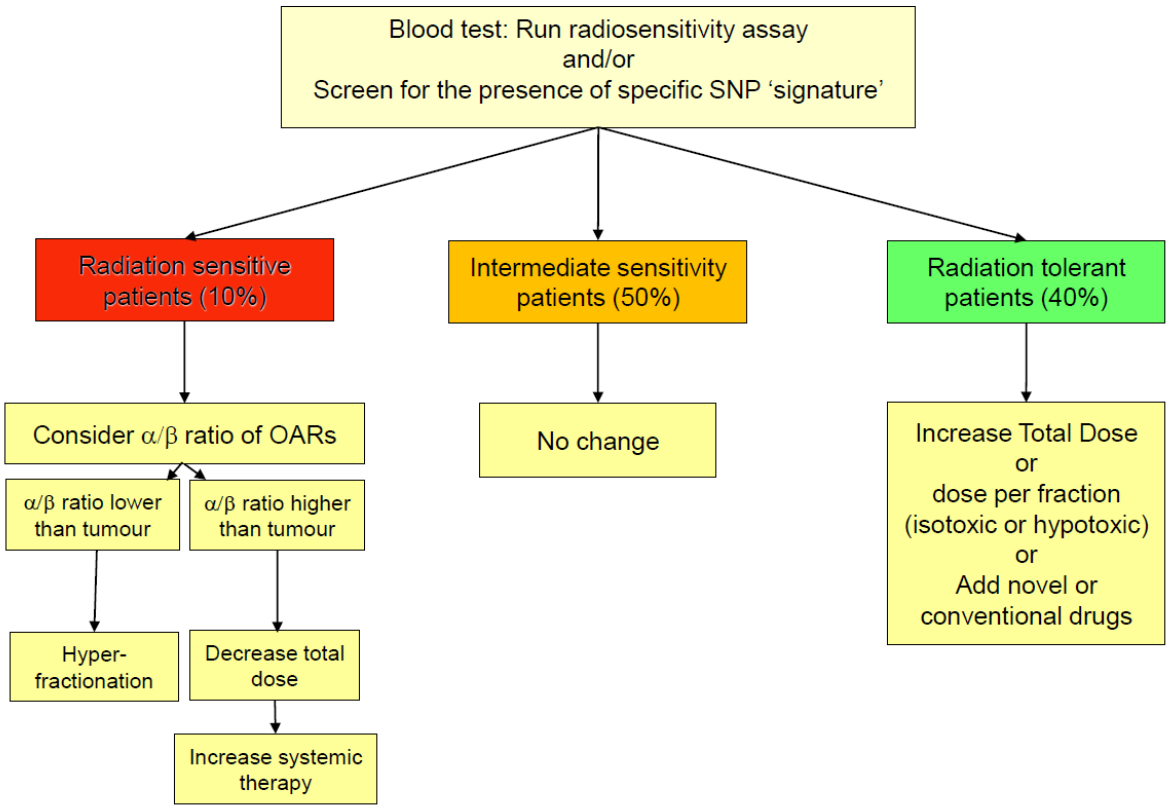

Figure 3. Schema for treatment alteration based on a predictive test of radiosensitivity. In principle this might be a radiosensitivity assay or the identification of a specific genetic 'signature'.

This shows the population of RT patients divided into 3 groups, which would allow change in management for half of the patients (sensitive 10% plus resistant 40%) receiving RT (see text for further details).

OAR = Organ At Risk.

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