
 COMMENTARY

Marriage of Surrogate Markers with Medium Term Tests for Carcinogenicity and Modification Potential

Nobuyuki Ito

Abstract

Given the immense variety of compounds being developed for introduction into the human environment, reliable medium term alternatives to traditional long term rodent test protocols for carcinogen risk assessment are a high priority. In vivo models are necessary because it has been well established that there is a lack of complete correlation between mutagenicity and carcinogenicity. Optimally, they should be able to detect not only complete carcinogenic or promoting potential, but also any ability to inhibit neoplasia. In order to be effective, they must take into account the detailed available knowledge on mechanisms of action of carcinogens and modulating agents. To allow shortening of the time period, attention must be concentrated on preneoplastic lesions and other surrogate. For the liver, a uniquely comprehensive set of background data have already been accumulated with the Ito model, for which, has a solid scientific basis, with quantitation of glutathione S transferase positive foci as the preneoplasia-based surrogate endpoint (PSE). A very practical candidate for routine application, its predictive power, flexibility and capacity to incorporate a range of mechanism-based surrogate endpoints (MSEs) can also provide a powerful tool for attainment of the twin goals of detecting carcinogenic agents and identifying promising chemopreventors.

Key words: Medium term tests- surrogate markers - PSEs - MSEs - animal models

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Medium-term Rat Liver Model

The importance of the environment and the chemical compounds to which human beings are exposed for cancer development is widely recognized (Doll, 1988). The need for strict control of agents presenting as carcinogenic risk factors, whether they operate by genotoxic or epigenetic mechanisms, is reflected in the complex regulatory systems which have become established in the developed world. However, traditional long-term test regimens to detect carcinogenicity are expensive in terms of financial and human resources, as well as time lost. This is a major hindrance to development and introduction of new pharmaceuticals and is the background to the high priority presently being awarded to establishment of alternative medium-term approaches. Their utility for carcinogenicity assessment was, in fact, one of the main themes at the recent Fourth International Conference on Harmonisation, attended by an expert working group responsible for drawing up guidelines for the regulatory bodies of the European Union, Japan and the USA. It was also the subject of extensive discussion at a special meeting at the International Agency for Research on Cancer in 1997. In vivo models and the end-point parameters which are applied must take into

account the large body of information available on mechanisms underlying tumor development, so that the aim of achieving the most reliable results in the shortest period of time may be realised. Given the increasing interest in identification of factors capable of inhibiting the processes leading to neoplasia, these factors should ideally allow both risk and benefit to be simultaneously assessed. The medium-term rat liver model developed by Ito and his group fulfills these criteria (Ito et al., 1997), and because of its reliability, practicality and the amount of background data that have already been generated, is a promising candidate for routine application.

Surrogate Parameters

Preneoplasia-Based Surrogate Markers (PSMs)

The long term test has benign and malignant tumors as its end points for decision making purposes. The nature of medium-term tests, with their relatively short duration, demands that appropriate surrogates be applied. The actual choice of parameters for measurement to allow a quantitative approach must be based on an understanding of the processes involved in the early stages of neoplasia. This extends from the initial exposure to a carcinogen at the single cell level

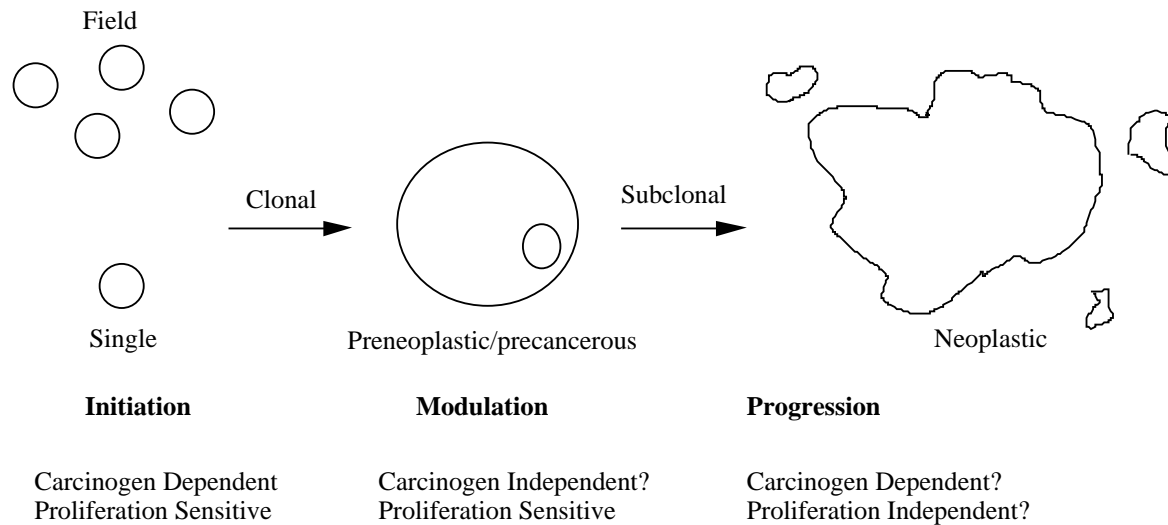


Figure 1. Stages in Carcinogenesis

through expansion by growth to the foci and nodules from which the final malignancies are generally considered to arise (see Fig 1 for a schematic illustration). Ideal surrogate end points should exhibit an absolute concordance with cancer development, factors causing their increase or decrease also resulting in a proportionally equal increase or

decrease in tumor yield (Einspahr et al., 1997; Schatzkin et al., 1996). Knowledge of the histogenesis of tumors in different organs has allowed identification of preneoplastic lesions, forming discrete foci or nodules, which may act as precursors for cancers. They are generally numerous and clearly they can not all give rise to neoplasia but, of the presently available markers, they appear to be the most directly correlated with tumor development (Bannasch, 1986). Therefore we propose the term preneoplasia-based surrogate end-points (PSE) as a yardstick for assessment of risk as well as benefit potential. With increase in size beyond the initiation stage, in addition to the obvious quantitative values, like numbers and size, these PSEs may also include qualitative characteristics, providing they correlate with eventual tumor yield (see Table 1 for a proposed classification of surrogate endpoints). For example, kinetic data for focal populations, like indices for proliferation or apoptosis, as well other phenotypic characteristics may be employed. The ability to generate mutations within preneoplastic populations would be an additional end-point of major significance.

Table 1. Proposed Classification of Surrogate Markers

INITIATION STAGE

- Preneoplasia-based surrogate endpoints (PSEs)**
Single cell/minifoci (e.g. GST-P immunohistochemistry)
- Mechanism-based surrogate endpoints (MSEs)**
DNA adducts (e.g. Immunohistochemistry/Blotting)
Proliferation (e.g. BrdU incorporation/ PCNA immunohistochemistry)

MODULATION STAGE

- Preneoplasia-based surrogate endpoints (PSEs)**
Quantitative data for foci (e.g. H&E staining/ GST-P immunohistochemistry)
Kinetic data for foci (e.g. Proliferation/Apoptosis/ Mitorelease)
Phenotypic data for foci (e.g. Histopathology/Enzyme or Molecular phenotype)
Genotypic data for foci (e.g. Mutations of growth control genes)
- Mechanism-based surrogate endpoints (MSEs)**
Quantitative data for tissue kinetics (e.g. Proliferation/ Mitoinhibition/Apoptosis)
Oxidation status (e.g. Lipid peroxidation)
Serum hormonal milieu (e.g. Insulin/Estrogen/ Testosterone levels)

Mechanism-based Surrogate Endpoints (MSEs)

With regard to parameters other than those dependent on preneoplastic lesions, there are a number of factors, acting either alone or in combination, which are well established to increase the likelihood of neoplasia occurring. While there is no obligatory relationship to cancer development, again pragmatic consideration dictates that they be accorded a certain degree of predictive potential. For example, in the prevailing paradigm, initiation of carcinogenesis requires both alteration at the DNA level and cell division to irreversibly fix genetic lesions so that they become heritable in preneoplastic cells. Such factors can thus be viewed as mechanism-based surrogate endpoints (MSEs) for initiation. Since for all practical purposes it is growth of preneoplastic populations which is necessary for attaining a sufficient

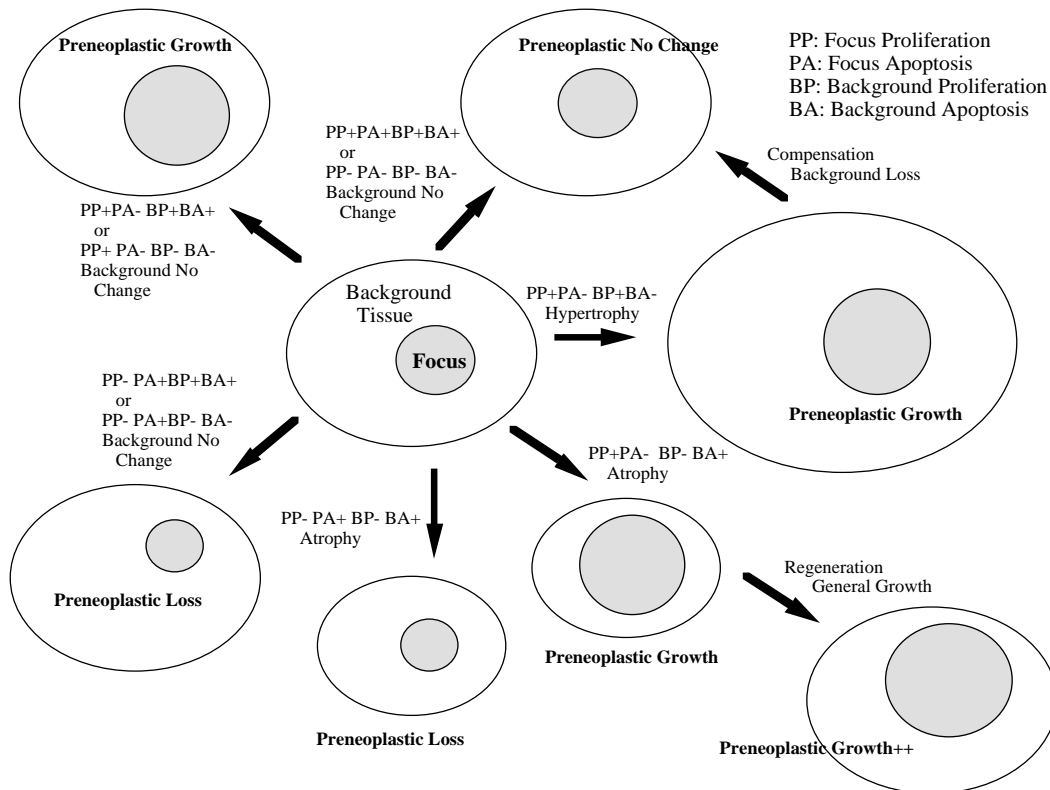


Figure 2. Balance of Apoptosis and Proliferation- Possible Outcomes

population size so that further genetic steps along the path to malignancy become probable, then the two determining phenomena, cell division and cell death, can be considered the most important MSEs for application in the post-initiation modulatory stage. The exact contribution of increased proliferation to tumor development continues to be a topic of lively discussion, but it is clear that chronic increase in the frequency of cell division is a major factor in human neoplasia. Exemplified by cirrhosis in the liver and colitis in the colon, the number of examples is so large as to preclude a comprehensive list in the present article. However, it has been estimated that over 15% of tumors worldwide are due to proliferation associated infectious-agents alone (Pisani et al., 1997), one good example currently attracting massive attention being *Helicobacter pylori* with reference to gastric cancer. However, in test assays, the correlation between carcinogenicity and toxicity or stimulation of proliferation has not been found to be absolute (Huff, 1992) and the existence of exceptions means that care must be taken in drawing conclusions. There is clearly no theoretical reason why proliferation by itself should cause neoplasia, as long as the regulatory machinery of the cell remains uncompromised, and the small intestine with one of the highest turnovers in the body is well known to exhibit a pronounced relative resistance to development of tumors. In this case the target cells for DNA damage and proliferation may differ so that all of the essential conditions for initiation to occur are lacking (Potten et al., 1992). There are obviously

many factors which can act at different stages of the processes underlying neoplasia, but there is a weight of evidence behind using proliferation as a parameter for screening (Ward et al., 1993; Cohen and Ellwein, 1991). As stressed by Farber (1995), however, it is the relative rates of proliferation within preneoplastic and background "normal" cells which determine how fast a lesion will grow. Therefore, the possibility that an agent could exert a promoting effect simply by inhibiting normal cells from dividing, the so-called mitoinhibitory influence well documented for many carcinogens (Farber, 1995), must be taken into account. Use of immunohistochemical techniques, for example to demonstrate incorporation of bromodeoxyuridine or the expression of proliferating nuclear antigen (PNCA), allows in situ examination so that in combination with markers of preneoplastic populations, both absolute and relative indices of growth can be generated. It is only recently that the potential importance of programmed single cell death or apoptosis has received emphasis (Chang et al., 1997, Stinchcombe et al., 1995). However, an awareness of the possibility that loss of responsiveness to the normal factors inducing this form of cell death can itself endow a major advantage to preneoplastic populations may also need to be incorporated as an essential component of testing approaches where sufficient preneoplastic populations are available for analysis.

With regard to other parameters which have been proposed for application as MSEs, lipid peroxidation or other

indicators of increased oxidative burden, including DNA adducts, may be of importance (Dreher and Junod, 1996). In addition, both experimental and epidemiological evidence has been obtained indicating that the hormones insulin and estrogen are possible endogenous promoting agents, for example in the colon and the female sex organs (Bruning et al., 1992; Giovannucci, 1995; McKeown- Eyssen, 1994; Moore et al., 1998), and therefore assessment of their serum levels might also be applied as an informative approach to detection of physiological changes conducive to neoplasia (Lagalopoulos et al., 1991, Tagliaferro et al., 1997).

For test purposes, significant increase in values for PSEs and MSEs are the hallmarks of initiators, complete carcinogens and promoting agents. The converse is the case for inhibitory agents. From the very early days of carcinogenesis research the liver, especially that of the rat, has been a major focus of attention, its large and relatively homogenous mass, sensitivity to a large proportion of known carcinogens, and well established histogenetic pathways leading to tumors (Bannasch, 1996; Bannasch et al, 1997) providing many advantages over other tissues. The fact that approximately 60% of compounds demonstrating carcinogenicity in long term tests include the liver among their target tissues is of particular significance for risk assessment (IARC Monographs). The biochemistry of the hepatocyte and its response to exogenous and endogenous agents are well documented and the availability of reliable markers for putative preneoplastic populations has facilitated experimental studies of carcinogenesis in the liver. In particular, the fact that expression of the glutathione S-transferase placental form (GST-P, GST-7-7) goes from

essentially nil to a large proportion of the protein production of the cell from very early stages after carcinogen exposure in putative initiated hepatocytes and minifoci (Moore et al., 1987; Satoh et al., 1989), means that quantitation is simplified, even single altered cells being reliably identifiable.

The actual protocol of our medium-term liver model is simple and there are no major practical difficulties involved in its performance. Building on the approach introduced for rapid induction of hepatocellular foci and nodules by Solt and Farber (1976), in its basic form (see Figure 3) it consists of an initiation step with a hepatocarcinogen, usually diethylnitrosamine at a dose of 200mg/kg, given as a single i.p. injection, followed after a recuperation period by exposure to the test compound for a period of 6 weeks. Two-thirds partial hepatectomy is performed at the end of week 3 to magnify any mitoinhibitory effects on normal hepatocytes and to provide a general growth stimulus. As documented in recent reviews (Ito et al., 1996;1997), the individual steps, like the choice of the initiator and its dose, the timing of the proliferation stimulus, which can also be achieved with administration of hepatocyte growth factor, and the length of the exposure period, have been investigated in great detail to maximise the predictive potential of the model using representative positive controls. In addition, a very strong correlation between yields of GST-P positive lesions, assayed in terms of number and size, and the eventual tumor incidence after long term administration of the same doses of carcinogens has been demonstrated (Ogiso et al., 1985). Conclusions as to risk potential are drawn on the

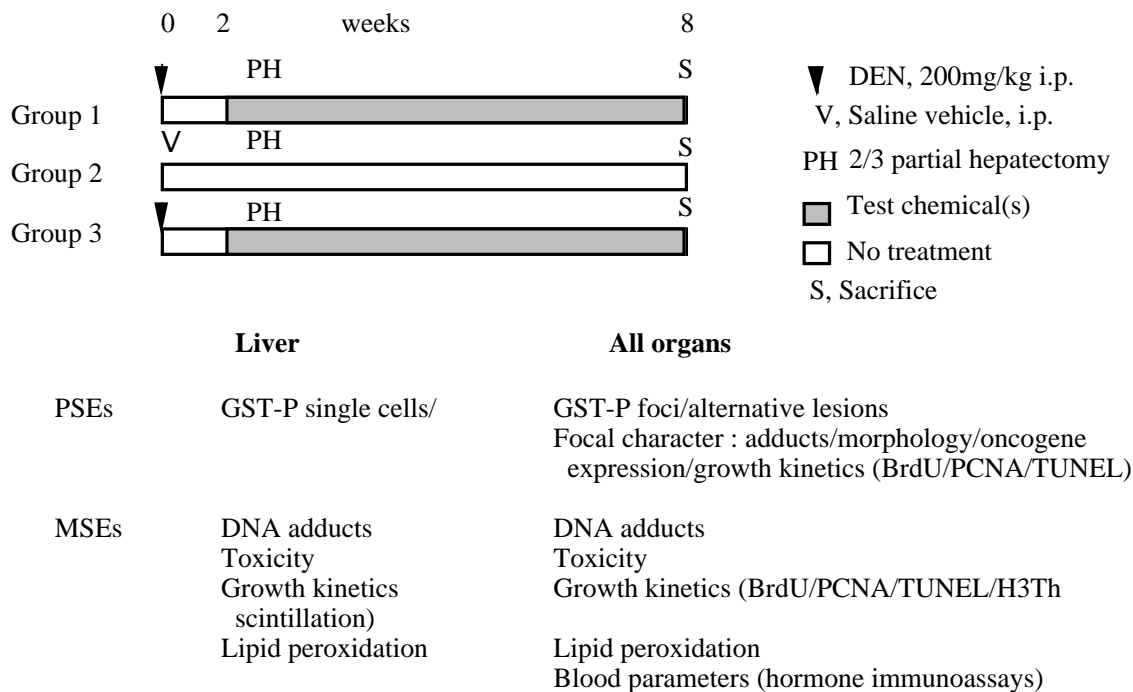


Figure 3. Protocol for the Ito Medium-term Liver Test

basis of comparison among groups 1 (initiation + test compound), 2 (initiation alone) and 3 (test compound alone). As argued by Cohen and Ellwin (1990), carcinogens can be divided into chemicals that cause genotoxicity and those which are non-genotoxic, at least in routine mutagenicity tests. The latter, so-called epigenetic, carcinogens appear to primarily act by causing proliferation. They can be subdivided into those which act physiologically, reacting with cell receptors and/or influencing signal transduction, cell cycle regulation and apoptosis, and those which cause toxicity and compensatory regeneration. The Ito model can distinguish between all three types (i.e. genotoxic, physiological non-genotoxic and toxic non-genotoxic). The first would be expected to generate preneoplastic lesions itself without the need for a prior application of carcinogen and can be detected on the basis of group 3 data. The others theoretically require prior initiation for generation of preneoplastic lesions, and therefore can only be detected in group 1, with the type of epigenetic agent being determined on the basis of whether markers of toxicity are positive. Such separation is clearly important in terms of dose dependence, with a threshold only deemed likely for agents depending on toxicity for their effects.

Using this model, a large number of compounds have now been investigated and about 90% of known hepatocarcinogens tested (97% of genotoxic, 84% of non-genotoxic and 100% of those with unknown genotoxicity) were positively identified. One important exception is the peroxisome proliferator group of compounds which appear to depress GST-P expression and induce preneoplastic lesions with an atypical phenotype (Rao et al., 1986). However, in these cases adoption of other PSE markers, for example histopathologically distinguishable foci, could be introduced as an alternative to the GST-P positive lesion (Metzger et al., 1995; Weber et al., 1988). Evidence of chemopreventive potential has also been generated for a large number of agents, many having antioxidant potential.

During establishment of the present model, attention was primarily concentrated on quantitation of foci. However, all of the other PSEs and MSEs listed in Table 1 could be readily applied with this protocol. The sampling points and the methods which could conceivably be employed to generate a detailed knowledge base are illustrated in Fig 3. The ability to generate DNA adducts is not routinely assayed although this could be incorporated by applying established techniques, but the inclusion of the PH step ensures that genotoxicity will lead to persistent mutations and presumably to initiation of carcinogenesis. Since the number of DEN induced lesions is high with the presently applied dose, only very strong initiators would be expected to cause a sufficient increase in the number of lesions for detection in group 1 but in the absence of this initiating step in group 2 the sensitivity of GST-P as a marker allows even weak carcinogenic agents to be identified. Toxicity, proliferation and apoptosis are assessable by the combined application

of histopathological and biochemical methods to tissue and blood samples obtained at week 8. Where necessary, the liver tissue taken at PH as well as blood samples could be used for detection of early stage influence.

With regard to carcinogens which do not normally include the liver within their target organs, the results so far have been equivocal. In its basic form the model is designed to assay for hepatocarcinogenic influence and therefore would not be expected to identify all carcinogens. However, the period of test compound exposure is sufficiently long for toxic or other effects on the parameters listed in Fig 1 to become apparent and assessment of other potential target tissues could readily be incorporated into the existing protocol. Finding of increased DNA adducts or altered proliferation kinetics (direct stimulation of cell turnover, mitoinhibitory effects on the normal tissue which would be expected to enhance growth of preneoplastic populations, or change in the apoptosis profile), and especially both, would provide very definite evidence of potential adverse effects, warranting further experimentation. Beneficial effects might similarly be identified, in terms of reduced lipid peroxidation or other positive biochemical indicators which might point to chemopreventive potential.

Recently, attention has been drawn to the possibility of adopting the same approach to identification of risk factors as well as possible preventive agents after wide spectrum initiation of carcinogenesis by application of a combination of chemical carcinogens targeting different organs or tissues (Hagiwara et al., 1993). The results so far obtained indicate that these wide-spectrum initiation protocols show promise. While the period necessary for initiation is relatively long at 4 weeks, and the test compounds must be given for a number of months they still offer advantages over long term testing. Furthermore, modification of the models to incorporate transplacental initiation with nitrosoureas, and using the natural growth phase to create conditions conducive to enhancing or inhibitory stimuli by exogenously applied test compounds is readily conceivable. Alternatively, other approaches to causing wide-spread proliferation in adult animals might be considered. One possibility is a starvation-refeeding approach, reported to enhance tumor development by increasing cell turnover in the liver and breast (Chiara et al., 1996; Hikita et al., 1997).

The scale of the task of ensuring that compounds introduced into the human environment are safe is huge. Any measures which can reduce the burden while maintaining standards of reliability are thus clearly welcome. The medium-term model described above is particularly appropriate since it allows promoters and inhibitors to be identified as well as complete carcinogens. This offers an improvement in efficiency and may indeed allow greater sensitivity than the long-term test to detect risk for agents which themselves have no initiation potential but do increase tumor yield by epigenetic mechanisms. The saving in time and resources means that such thorny problems as the effects

of combinations of compounds at low doses can be assessed. The Ito model has already proved invaluable for detecting summative and synergistic impact of heterocyclic amines, important environmental contaminants which are likely carcinogens in man (Futakuchi et al., 1996; Hasegawa et al., 1996).

Since we are not yet in a position to predict whether the influence of a compound in the medium-term will be precisely the same as that when chronically administered for very long periods of time, it is not proposed that long-term 2 year rodent studies for detection of carcinogenicity in vivo could be completely dispensed with in the foreseeable future. Nevertheless, a judicious combination of short-term mutagenicity and medium-term tests based on knowledge of the mechanisms underlying preneoplastic lesion development and relative indices of proliferation and apoptosis provides a pragmatic approach to initial screening so that preliminary conclusions can be drawn. Where obvious enhancing effects are evident, above those of positive controls selected for each organ of interest on the basis of available human and experimental findings, then a strong recommendation for classification as a carcinogen could be made. Only under exceptional circumstances would any further animal experimentation be warranted. In the more problematic case of negative or unequivocal findings, then confirmation by a single 2 year study, most likely in the rat, might be the logical outcome. The actual strain might vary with the organ(s) of interest to take into account similarities with the human case in terms of metabolism and other relevant factors. Use of transgenic/gene targeted rodents might be advantageous in some situations. Where conceivable, long-term rodent studies should also concentrate attention not just on lesion development but on a range of surrogate parameters to build up a data base for an optimized interactive knowledge-based approach (Ashby et al., 1994; Butterworth et al., 1995).

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Personal profile: Nobuyuki Ito

Professor Nobuyuki Ito is so well known in the International world of Toxicological Pathology that even a few words would be superfluous.



Suffice it to say that, since moving from Nara, he has established Nagoya City University as a center of excellence for Toxicological Pathology and made an incomparable contribution to cancer prevention.