Chapter 3

THE SEARCH FOR GENES WHICH INFLUENCE PROSTATE CANCER METASTASIS: A MOVING TARGET?

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Abstract: The process of cancer and prostate cancer metastasis is complex and requires fundamental changes to the behaviour of the parent cell. While the stage at which essential mutations for prostate cancer metastasis occur remains controversial, it is likely, based on current evidence, that an accumulation of genetic damage is required. However, the study of cancer metastasis is clearly dependent on the availability of suitable *in vitro* and *in vivo* models. Not every model represents the full *in vivo* situation in man, but a combination of these models is now becoming available in prostate cancer and should allow a more detailed assessment of the specific genes involved in metastasis and the preferential adhesion in bone. Identification of specific genes associated with particular pathology has also taken tremendous steps forward in the last few years. Differential expression analysis, of both the RNA and also protein levels are providing new targets for therapy, specifically directed against metastatic disease. However, for longer term prospects the ability to detect metastasis in a simple blood sample would offer the most hope of permanent treatment or indeed cure. Based on serum profiling, such methods should soon be available to the oncologist in the clinic. On-line catalogues of genes whose expression is perturbed in metastatic processes offer the first clues to the key events in this complex biological process. It is perhaps from these catalogues improved animal models and indeed the more global analysis of patient samples from bio-banks that the key events and a genetic basis will be identified.

Key words: prostate cancer metastasis, genomics, proteomics, *in vitro* tests

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1. INTRODUCTION

Without metastasis, prostate cancer would be both tolerable and treatable. The high incidence of indolent and organ confined disease is testament to this sweeping generalisation. Equally, if molecular markers of metastatic spread can be identified, then the choice of treatment for many patients would be easier and more radical, even curative. However, should prevention and treatment of the primary tumors prove difficult or impossible, then a knowledge of the phenotype of advanced metastatic tumors should allow us to target these lesions for destruction by conventional (drug based) or more innovative means such as gene and/or immunotherapy (1).

The process of metastasis has been reviewed many times (e.g., 2) and has been subdivided for ease of analysis into a number of discrete stages (see Figure 1). It has been suggested that at least 10 separate genetic

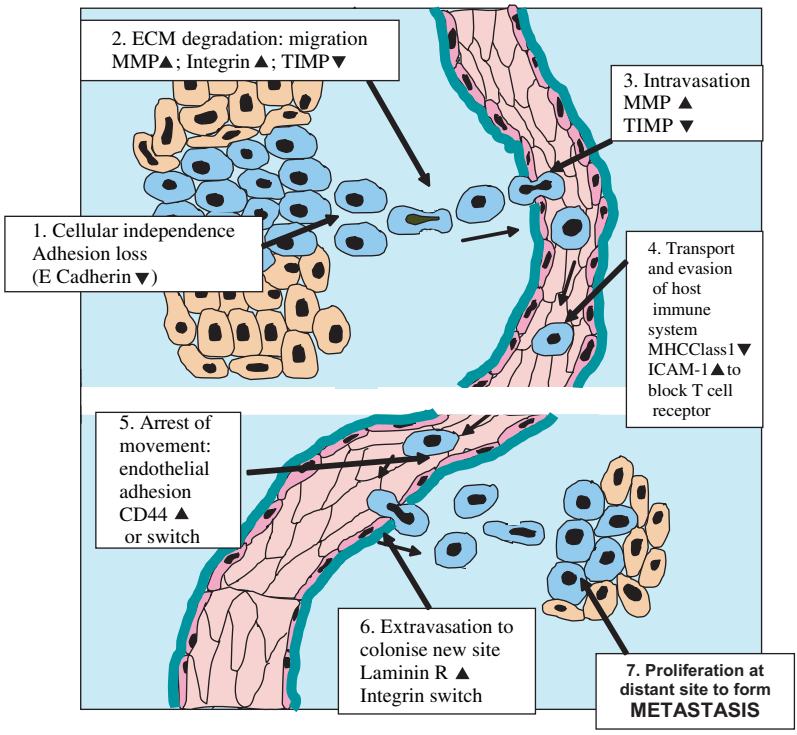

Figure 1. Stages in prostate cancer metastasis. Basic processes in tumor metastases are indicated in the boxes with some key changes in gene expression indicated at each stage by the solid arrows.

alterations and/or genetic selections could be required to permit establishment of a tumor at an extra-prostatic site (3), and many investigations have concentrated on defining a role for genes of known function within the metastatic process. Thus demarcations in the stages of metastasis development as shown in Figure 1, and the primary consequences of the changes at each stage have dominated thinking on the types of genes investigated. Perhaps the complexity of the phenomenon demands a more even-handed and unbiased investigation, now possible in the days of whole genome analysis, and mass proteomics. To provide an alternative approach, I have adopted a technology-based approach to prostate cancer, and will seek to justify the inevitable preferences and prejudices about the significance of the many metastatic markers, more on the basis of genetic preference (4) rather than a seed and soil (5) approach. As metastasis is a basic property of most tumor types, at an advanced stage, then it is likely that many of the basic parameters will be shared (as outlined in Figure 1), but the behaviour of prostate tumors is sufficiently different even from breast cancer at the metastatic site, for example in its osteoblastic nature, compared to the osteolytic properties of breast carcinoma (reviewed in 6) to suggest the existence of certain unique features. For this reason, I have also deliberately eliminated a detailed discussion of angiogenesis, as one of the most basic necessary and important steps in metastatic escape. The subject has been reviewed in great detail, and I would direct the interested reader to reviews on this topic such as Folkman (7).

2. GENETIC ALTERATIONS: A MATTER OF PERMANENCE

Cancer is normally associated with a loss of genetic information i.e., a dominant recessive disease, and it has long been supposed that metastasis occurs as a result of removal or suppression of “metastasis suppressors”. However, there is also good evidence for metastasis activators or enhancers, where a gene is expressed either aberrantly or at higher levels than normal. Genome wide expression screens have identified candidates of both positively and negatively acting gene groups (see below).

What is perhaps more important however, are the mechanisms by which the altered levels of expression of the two types of gene are ultimately obtained. These are outlined in diagrammatic form in Figure 2. Firstly, for autosomal *suppressor* genes, one or both copies can be inactivated by deletion or mutation in the classical tumor suppressor gene mechanism, as shown in Figure 2a. Most frequently one of the two

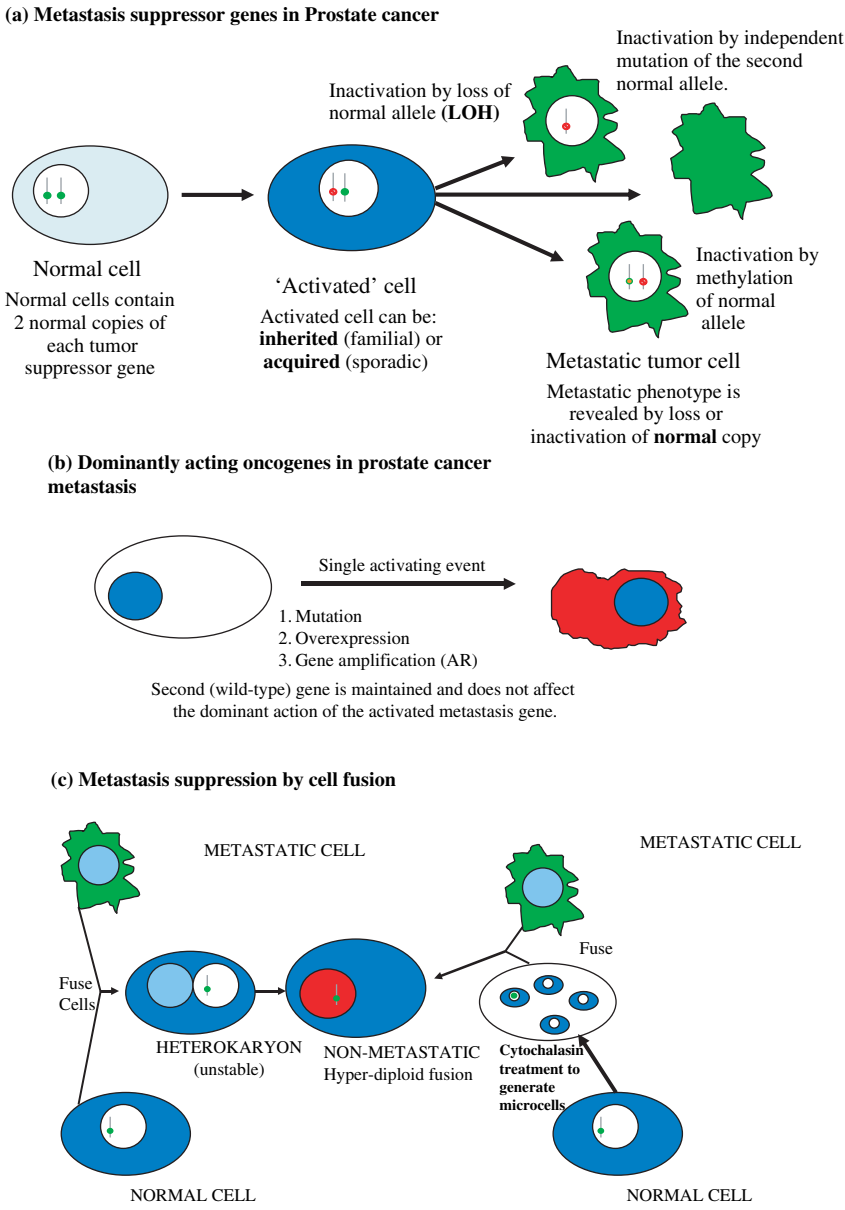

Figure 2. Human metastasis genes. **A:** The principles behind metastasis suppressor genes. The three methods of inactivation of a normal suppressor allele are illustrated. **B:** Dominantly acting metatasis enhancers, a subset of known oncogenes act by direct over expression, either in a wild-type or constitutively active mutant form to promote tumor spread. **C:** Identification of metastasis suppressor gene loci by cell fusion or direct chromosomal introduction into indicator metastatic cells.

alleles is deleted, whilst the remaining allele is silenced by epigenetic or mutational mechanisms. For a sex-linked gene in males, the situation is more straightforward, with a single gene inactivation required to abolish expression. A good example of this is the gene for androgen receptor, which is located on the X chromosome. Chromosomal alterations leading to over-expression of a metastasis *activator* are easier to explain, with both intra-chromosomal tandem duplications and minute chromosomes observed containing amplicons of both the metastasis activator and closely linked genes (Figure 2b). This latter co-amplification can result in false identification of implicated genes.

An increasingly common mechanism of changing gene expression levels is epigenetic alteration. A full description of the various intra-nuclear modifications, which lead to activation or silencing can be found elsewhere (8). The most commonly recognised and observed mechanism for selective gene silencing is CpG methylation in promoter and enhancer regions associated with the gene in question. The great advantage in changes of this type is their reversibility. For a (cancer) cell to survive *adaptability* must be a major advantage, and the permanent changes in gene expression produced by the various chromosomal alterations would be a poor strategy in a hostile environment, for example when establishing residence in an extra-prostatic site.

3. BIOLOGICAL ASSAY SYSTEMS TO STUDY GENE FUNCTION IN PROSTATE CANCER METASTASIS

In order to carry out detailed genetic analysis of the genes, which are activated in *metastasis* of prostate cancer, quite distinctly from genes activated in prostate cancer, the choice of biological material is paramount. The old adage about the quality of the input matching the output is particularly true here. The source of the material has ranged from rodent models through to material dissected from clinical material. All have produced candidate genes, but in the case of rodent models, their relevance to human disease has in some cases yet to be confirmed.

3.1 *In vitro* Approaches

Considerable experimental work has been conducted on the standard prostate cancer cell lines as an attempt to analyse metastasis. The PC3 cell line was originally isolated from a bone marrow metastasis, LNCaP

is derived from a lymph node metastasis and DU145 from a brain metastasis (an extremely rare occurrence for prostate cancer) (reviewed in 9). Comparisons between these cell lines have been made, in attempts to define both site-specific changes, and androgen sensitivity of genes up-regulated in metastasis. There are considerable shortcomings in this approach, particularly as the different cell types and cultures derived from normal tissue, such as the PNT series (9), are all from different patients, and were established in culture using different methods. After many years in culture, they have become grossly aneuploid and also heterogeneous. Unless cross-related to the tissue arrays (or similar) as discussed later, these simple models only offer a small fragment of the metastasis story.

A better approach would be to use malignant variants of the same cell type. Most cell lines do throw off variants in both culture and *in vivo* selection (for example the multiple xenograft variants reviewed in van Weerden and Romijn, 10). Comparisons of non-malignant cells "progressed" by treatment with chemical and viral carcinogens offer the controlled baseline for comprehensive analysis of metastatic changes. One such comparison was reported by Hukku et al. (11) who analysed the multiple genetic changes that occurred when a non-malignant HPV18-immortalised cell line, progressed to malignancy as judged by the ability to form tumors in severe combined immunodeficient (SCID) mice. However, no analysis of metastasis was presented, although the model should lend itself to such studies.

3.2 Cell Fusion/Single Chromosome Transfer

As long ago as 1969, Harris et al. (12) showed that fusion of malignant cells with non-malignant cells would result in a non-malignant phenotype. Further experiments by Sidebottom and Clark (13) extended this "suppressor gene" hypothesis to metastasis (in the chosen cell type at least) implying that metastasis is a recessive disease. The assay was taken forward by Stanbridge, who used the dominance of the murine karyotype over human chromosomes in murine:human cell hybrids to produce stable murine cell lines with relatively few human chromosomes. Suppression of the malignancy of the murine parent in the hybrid indicated that the retained human chromosome contained one or more suppressor gene (14). To further improve the technology, a series of murine cell lines, each containing a single human chromosome (and a drug selection marker to permit primary selection) were developed. These systems have been successfully employed to map senescence genes and other tumor suppressor, and formed the basis of metastasis suppressor identification in the Dunning model (e.g., Mashimo et al., 15)

which is described in more detail below. The principles of cell fusion mediated suppression of metastasis are illustrated in Figure 2c.

The resources of the Human Genome Program, now offer bacterial artificial chromosomes and cosmid clones which span the entire genome in manageable segments, to permit more precise "biological" mapping. While these whole chromosome methods may seem crude, they have the major advantage of transferring gene clusters and associated genes, while overcoming single gene silencing (often observed in integrative gene transfers) by transfer of a large genomic segment.

However, the biological assays all require a means of quantifying metastasis (e.g., 16). Perhaps the simplest approach is to measure the ability of cells to form viable colonies in semi-solid support medium; this is a strong indicator of independence from normal cellular controls, and interdependence between stromal and epithelial cells for example. A more precise measure of invasion in metastasis is the long established chamber assay, where the tumor cells are layered on top of a matrix (frequently collagen or matrigel for example), prepared in a cylindrical insert for a tissue culture chamber. By comparison with a known metastatic cell, the rate at which the test cell migrates through the matrix is measured by crystal violet staining of the distal surface of the membrane after various time points. Most cells will eventually penetrate such matrices, but truly metastatic cells will appear within 24 hours, at the distal surface, having penetrated and frequently digested the matrix. There are variants on this procedure:

(i) Embedding of stromal cells in the matrix can provide both positive and negative stimuli to invasion and

(ii) Introduction of a layer of endothelial cells can simulate the essential penetration of microvasculature as a first step in migration out of a tissue such as prostate.

3.3 Motility Assays in Two Dimensions

In vitro analysis of metastatic tumor cells, have provided good evidence that the most metastatic cells display greatly altered motility and cellular organisation. The original model for this was the 3T3, 3T6 and 3T12 embryonic mouse cells. The highly tumorigenic 3T12 cells displaying all of the properties of a true tumor, while 3T3 cells remained 'normal' with elements of growth control and special regulation. Quantification of *motility* remains problematical. Some guidelines were provided by Mohler et al. (17) studying the Rat Dunning system (see above), and this can be translated into studies on prostate epithelium, either in mono-culture or in tissue recombinations (16). Since different tumor cells exhibit different sets of

properties almost certainly defined by genetic changes, a combination of scores for ruffling, pseudopodial movement, translative movement results in an overall motility index, which correlates well in most (but not all) cell lines with metastatic ability (Table 1) (18). In combination experiments, prostatic stroma positively regulated motility, while in most cases not affecting growth rates (16).

The significance of this reductionist approach to metastasis has recently been given extra credibility by microarray studies on metastatic human and mouse melanoma cells (19, 20). Using the mouse techniques described above, metastatic variants were selected and screened by 7k cDNA microarray. This biologically sound approach, which reduced the extreme "noise" often seen in such experiments, resulted in over-expression of fewer than 20 genes, but no significant fingerprint for metastasis in melanoma. As shown by Kozlowski et al. (21), there are independent routes to and origins for a metastatic phenotype, and the gene profiling simply provided sound evidence for this 20 year old hypothesis. However, the expression of 3 genes: RhoC, 1fibronectin and thymosin β4 was elevated in all of the mouse and human metastases (19). Fibronectin has been linked to cell migration, as it is a component of the extracellular matrix and was a common upregulation product in both recent studies on melanoma. Thymosin β4, like other thymosins, binds to monomeric actin, sequestering it and preventing polymerisation into fibres, and as a result reduces cellular motility via lamellipodial extension (22). Here there is a clear relationship to prostate cancer, where earlier studies on the Rat Dunning model revealed Thymosin β15 as an over expression product in metastatic cells, while anti-sense inhibition of Thymosin β15 in metastatic cells prevented metastasis (23).

Table 1. Quantification of prostate epithelial cell motility as a determinant of invasiveness

Motile property	**Scoring system**					
	Score:	**0**	**1**	**2**	**3**	**4**
Ruffling			None/little	Average	High	
Pseudopodia		None	1–49%	50%	51–99%	100%
	Score:	**0**	**1**	**2**	**3**	**+1***	**+2***
Translation		None	Little movement as a tight colony	High movement as a tight colony	Movement as a scattered colony	<50% of cells show individual translation	>50% of cells show individual translation

*The extra scores for individual translation are added to the basic scores of 0–3 for translation

Finally, overexpression of RhoC (a GTP-hydrolysing protein like ras) has also been shown to affect cell migration (24). However the microarray analysis failed to detect changes in Rho A and B, and only re-introduction and overexpression of RhoC was able to convert non-metastatic melanoma to a metastatic phenotype. No direct involvement of RhoC in prostate cancer invasion has been reported in a recent comprehensive review (25), although Rho kinase inhibitors appear to suppress malignancy in experimental PC3 models of carcinoma of the prostats (CaP) (26).

4. SYNGENEIC MODELS OF PROSTATE CANCER

4.1 Rat Dunning Model

Amongst the first models to be exploited was the Rat Dunning carcinoma, which exists as metastatic and non-metastatic variants (e.g., AT2.1 and AT3.1). Cell fusion experiments between the variants resulted in a non-metastatic heterokaryon (27). The Dunning metastatic cells were therefore used as an indicator system for similar metastasis suppressor genes, leading to the identification of firstly loci at 8p, 10q and 11p 17p and later positive identification of the metastasis suppressor genes KAI1 at 11p12 and a role for CD44 (at 11p13) and MAPK kinase 4 (at 17p11.2)

4.2 Mouse Prostate Reconstitution Model

The ability to derive differentiated prostate tissue from reconstitution of individual cell types has provided powerful tools for the study of glandular development for more than 30 years (28). More recently Thompson and co-workers were able to use a similar model to investigate carcinogenesis in the mouse prostate by transfections of dominantly acting oncogenes into the epithelial component of the reconstitution (29). The model has been further refined by the use of p53 knockout mice as the recipient of the myc and ras oncogenes, which resulted in development of micro-metastases in bone and other tissue (30), implying a critical role for p53 in metastatic development in this system, paralleling the demonstration of p53 mutants in metastatic human prostate cancer (31). The model has therefore generated the correct, genetically matched background, from which genetic lesions can be assessed (reviewed in Thompson et al., 32). Using primary tumor and lung metastases for example, and differential display (see below) a role for overexpression of Caveolin 1, which is present in membrane invaginations

responsible for small molecule transport, urokinase signalling and with integrin mediated signalling has been postulated (reviewed by Bangma et al., 33). Caveolin expression is undetectable in normal human prostatic epithelium, but appears to be upregulated in human metastatic tumors (34).

4.3 Transgenic Mouse Models

Considerable investment has been made in murine models of human cancers (35). For gene isolation and functional characterisation, the mouse is closely related to man, and is clearly a better experimental model for investigation of molecular therapies, in particular immune therapy for metastatic disease. However, the relatively short lifespan of the mouse probably prevents the accumulation of necessary mutations to spontaneously develop prostate cancer. Indeed the mouse is more likely to die of other tumor types. There are a number of other significant biological differences, which promote some caution in the extrapolation of the mouse situation to humans:

Firstly, the murine prostate atrophies with age, in contrast to human prostate, in which hypertrophy is observed. Secondly, the mouse prostate has 3 lobes, in direct contrast to the alobular human prostate, which is a single gland composed of transitional, peripheral and central zones.

To produce prostate cancer in mice requires tissue specific expression of a strong oncogene from a tissue-specific promoter (36). Not all of the models however produce metastatic disease. The TRAMP model (probasin promoter driven SV40 T antigen, (37)), results in tumors in the dorsolateral lobe (murine equivalent of the peripheral zone) which metastasise to lymph node, lung and (in the correct genetic background) to bone. However, the probasin promoter is active in luminal cells of the murine prostate, and most human prostate cancers probably arise from the basal epithelium. The genetic changes observed are however similar if not identical to human disease, including the loss of E-cadherin expression. The TRAMP model also allows derivation of individual cell lines, which behave in a predictable and similar manner to the original Tag –induced tumors when transplanted into syngeneic hosts (38) and should also form the ideal raw material for gene identification. For example, expression of the murine homologues of a number of human prostate carcinogenesis-associated genes is frequently observed (39). TRAMP C2 metastatic lesions can be induced by abrogation of transforming growth factor beta (TGF-β) responses in bone marrow (40) and re-expression of maspin (one of the most common negatively regulated genes found in human CaP by microarray) also leads to reduced metastatic potential (41).

Other models using dominant oncogenes, which are capable of producing metastases include (i) the C3(1) driven T antigen (42) and (ii) the murine

cryptdin (CR2) driven T antigen model (43), which differs from the other in that it targets the neuroendocrine cell component of prostate.

As our knowledge of the genes deleted during prostatic carcinogenesis increases, the specific deletions in tumor suppressors can be modelled in transgenic knockout mice. Two good examples of such tumor suppressor genes, where loss of heterozygosity is frequently observed in human carcinoma are PTEN (chromosome 10q23) and Nkx3.1 (chromosome 8p21). Single knockout mice in Nkx3.1 develop lesions perhaps akin to prostatic intraepithelial neoplasia (PIN) (44), whereas PTEN knockout mice develop adenocarcinomas in multiple tissues (45). The addition of a p27 KIP1 cyclin dependent kinase inhibitor knockout on the PTEN background results in prostate carcinogenesis. The triple knockout results in true carcinogenesis, which should further progress to metastasis in the absence of the viral oncogenes used in the TRAMP and similar models. Development of these systems should allow functional characterisation of metastasis candidate genes, and development of therapies, in a prostate-specific manner.

4.4 Xenograft in Nude/SCID Mice

The nude (athymic) and now the severe combined immunodeficient mouse systems, offer the ability to culture a range of human tumor types, both by inoculation of established tumor cell lines (21) and direct graft of tumor tissues such as CWR22 (46). The range of systems available was reviewed recently by van Weerden and Romijn (10).

Again the system makes compromises, as the lack of a functional immune system can affect both location and take rate of the grafted tumors. One solution to this was described by Nemeth et al. (47), who engrafted human tumor cells located within macroscopic human bone fragments into SCID mice, to study the metastatic lesion in its ultimate environment. In this model, only PC3 (originally from a bone metastasis) was able to colonise bone fragments after intravenous injection, whereas *all* of the tumor cells injected showed an ability to grow when grafted in human but not murine bone. Significantly, evidence of intense stromal:epithelial interaction was observed in the human:human grafts. In contrast to the majority of natural human tumors which are osteoblastic, the grafted cell lines produced ostolytic growth.

The PC3 cell is much favoured in studies of this type, as sub-lines with particular metastatic abilities can be readily generated (48, 49) by orthotopic inoculation. However, the LNCaP (androgen responsive) cell

line, derived originally from a lymph node metastasis, can also be used to generate androgen independent and specifically metastatic sublines (50). These cell variants provide homogeneous sources of material for gene isolation and functional analysis, although in all cases the ability of the cells to colonise the metastatic sites can be modulated by the presence of human stroma (51).

Most recently new sublines have been developed which express fluorescent markers such as GFP (52) and luciferase (53), which offer the added advantage of real time monitoring of the metastatic process.

To identify metastasis associated genes, sublines of the CWR22 xenograft have been employed. Multiple variants are now available, including androgen independent (54) and highly metastatic cells (55) which have been directly employed in microarray analyses to identify implicated genes such as S100P, whose role in prostate tumorigenesis has been suggested in other studies (56). Although the S100P gene is androgen regulated, linkage of over-expression to both androgen independent and recurrent, metastatic disease was observed by Mousses et al. (55). Other members of the calcium and magnesium binding protein family of S100 proteins such as S100A4 have been linked directly to metastatic disease ((57, 58) and I Bronstein, unpublished results), which suggests that the metastatic role could predominate.

Lastly, and possibly of relevance to the metastatic process in prostate, is a study of skin carcinogenesis in the mouse. Detailed study of the changes in signalling pathways in clonally derived skin carcinoma cell lines (59) confirmed that, after initiation by H-ras activation, over-expression or gene amplification (only rarely seen in human prostate cancers) TGF-β signalling induces an epithelial/mesenchymal transition (overexpression observed in metastatic human prostate cancers). Phosphatidylinositol-3 kinase activation, which is observed as a result of PTEN inactivation in CaP ((60) and M Sharrard, personal communication), can also co-operate to inhibit TGF-β induced apoptosis. The key downstream event for TGF-β signalling is now the activation state of the SMAD 2 and 3 proteins: ie whether they are phosphorylated and nuclear in location, resulting in upregulation of SMAD transcription control targets (61). Such activation has been observed in CaP (62), and is a key player in the increased migratory capacity (via structural changes to the cellular cytoskeleton), rather than the proliferative capacity (via abrogation of cell cycle controls) of the tumor cells. This data, and its obvious application to carcinoma of the prostate provides further strong *in vivo* and *in vitro* evidence for the importance of the epithelial:mesenchymal transition in the development of metastatic potential (63).

4.5 Primary Tissue Comparisons: Tissue Microarrays

The data reported by Mousses et al. (55), see previous section) was given added relevance to native disease by validation using in situ hybridization and immuno-histochemistry on tissue microarrays, in which 60% of metastatic tumor samples over-expressed S100P. In contrast to many of the earlier studies, where statistical relevance was always hampered by the need for multiple immuno-staining, or in situ hybridization to many individual sections of tissue. The advent of a tissue microarray, in which many different types of pathological disorder are represented on a single slide from multiple patients (64) can produce significant data in a single reproducible experiment, which also can take account of the heterogeneity of the prostate cancer phenotype. It is useful not only for determining intracellular location of proteins whose expression is changed in metastasis, for example in the DNA micro-array study of Dhanasekaran et al. (65), who confirmed hepsin and the serine-threonine kinase pim-1 over expression, but also in the analysis of gene copy number. Interphase cytogenetics, to detect amplified and translocated genes in prostate cancers have been instrumental in confirming the amplification of the AR and c-myc genes in a single survey of 371 specimens (66) and have now become an essential tool in providing clinical relevance for candidate genes. The various chromosomal loci and specific metastasis genes, which have been identified by direct application of cellular and animal models are listed in Table 2 and illustrated on an ideogram of the normal human karyotype in Figure 3a.

5. IDENTIFICATION OF GENES

In order to study metastasis genes the *techniques* remain the most critical decision. Perhaps the easiest approach taken is to simply test the deletion/amplification status and expression patterns of genes implicated in the metastasis of other more easily studied tumor types. This is not the most imaginative approach, but given the ubiquitous nature of most of the processes involved in metastasis it has been very popular, although the choice of biological material is variable. As I shall demonstrate, the most recent exploitation of the database provided by the human genome mapping program is a fingerprint of metastasis that is tumor type independent, but may have significant consequences for tumor diagnosis and therapy.

Table 2. Chromosomal changes implicated in Human prostate Cancer Metastasis: Cell and Animal models

Source of metastases	Gene	Chromosomal locus	Activator (+) or suppressor (−)	Method(s) of isolation	Reference(s)
PC3M	?	1q21-22,	(+)	CGH	(67)
	PTEN	10q23-ter,	(−)		
	?	18q12-21	(−)		
LNCaP	BRCA2 ?	13q12-13	(+)	CGH	(67)
	?	16q23-ter	(−)		
	?	16q21	(−)		
Dunning Model	KAI 1(CD82)	11p11.2	(−)	Microcell Fusion	(109)
	CD44	11p13	(−)		(110)
	MAPK kinase	17p11.2	(−)		
	4	2p22-25	(−)		
	?	7q21-27/7q31.2-32	(−)		
	?	8p21	(−)		
	?	10cen-q23	(−)		
	?	12cen-q13/12q24-ter	(−)		
	?	16q24.2	(−)		
	?	20p11.23	(−)		
	?	Yq11.1/	(+)		
	Thymosin β15				
Mouse prostate reconstitution	Caveolin	7q31.1	(+)	DD	(34)
(myc/ras/p53)	p53	17p13.1	(−)		(32)

Table 2. (Continued)

Melanoma 'fingerprints'	Rho A/C	3p21/1p31.1	(+)	Microarray	(19)
	Fibronectin	2q34	(+)		
	Thymosinβ4	Xq21	(+)		
TRAMP	TGF-β	19q13.1	(+)	Various	(40)
	E-cadherin	18q21.3	(−)		(41)
		16q	(−)		(39)
					(45)
					(44)
Compound Knockout mice	PTEN	10q23	(−)		(35)
	Nkx3.1	8p21	(−)		
	P27KIP1	12p13.1	(−)		
CWR22 (orthotopic mouse)	S100P	4p16	(+)	Microarray	(55)

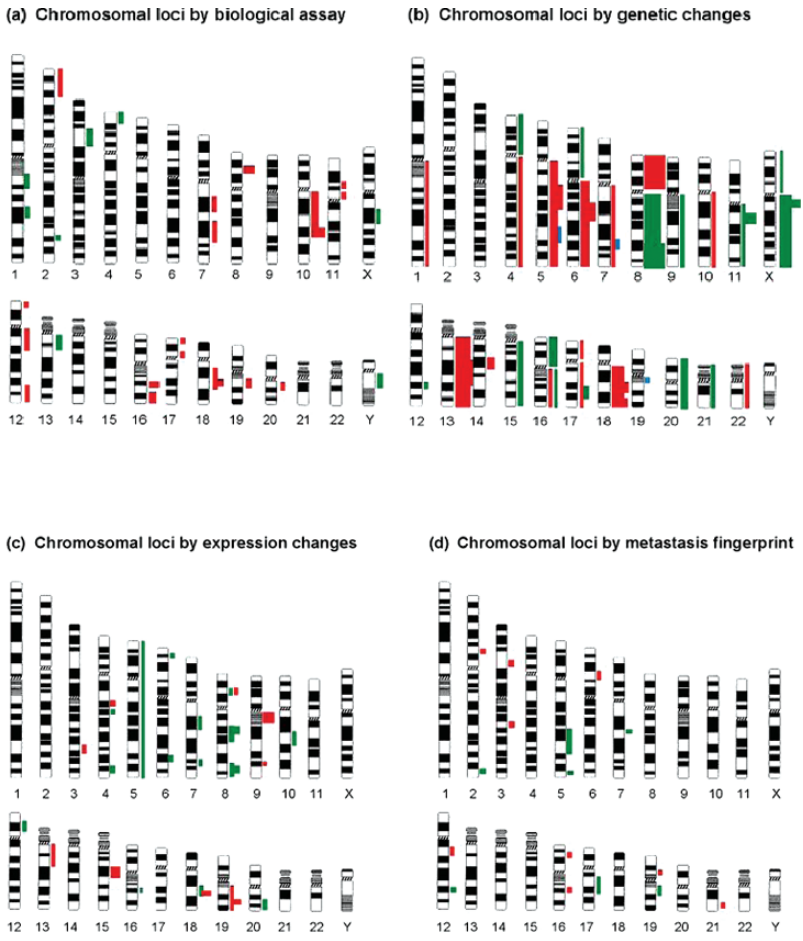

Figure 3. Chromosomal locations of genetic changes observed in prostate cancer metastases. Data is detailed in the text and in Tables 2–5. Gains (gene amplifications) are indicated by green bars and losses by red bars. The frequency of changes are denoted by the thickness of the bars.

6. TECHNIQUES TO IDENTIFY GENE LOSS AND AMPLIFICATION

6.1 Cytogenetics/Comparative Genomic Hybridization

Numerous cytogenetic abnormalities have been shown in prostate cancers, although consistent data has been elusive. Most tumors appear on

Table 3. Chromosomal changes implicated in Prostate Cancer Metastasis: Genetic Analyses of Human Tissues

Source of metastases	**Gene**	**Chromosomal locus**	**Activator (+) or suppressor(−)**	**Method(s) of isolation**	**Reference(s)**
Human metastatic tissue relative to intraprostatic disease		18q21 18q22-23	(−) (−)	LOH	(111)
Human lymph node and bone metastases		8q 8p	(+) (−)	CGH / Cytogenetics/ Interphase *in situ* hybridization	(112)
Linkage analysis in brothers with CaP		5q31-33 7q32 19q12	?	Linkage analysis	(71)
Human tissue microarrays	Androgen receptor c-myc	X 8q	(+) (+)	FISH/tissue microarrays	(66)
Human Tissues		*5q(1), 6q(1), 7q(1), 8p(4), 10q(1), 13q(1) 16q(1), 17p(1), 17q(2) 18q(3)	(−)	LOH	Reviewed in 113

Table 3. (Continued)

Source of metastases	Gene	Chromosomal locus	Activator (+) or suppressor(−)	Method(s) of isolation	Reference(s)
Human bone marrow metastatic deposits		8p, 13q, 18q 8q, 9q, 20, X	(−) (+)	M-FISH and CGH	(114)
"Advanced" prostate cancer		1q, 4q, 5q14-21, 6q16.1-21, 13q21.3-22, 14q21, 22 4p, 6p, 8q24.1-24.3, 11q, 12q23, 15q, 16p, 17q23-24, 20,21	(−) (+)	FISH and CGH	(115)
"Advaned" prostate cancer	AR c-myc cyclin D1	8p, 13q, 6q, 18q, 5q (2q, 4q, 16q) 8q, Xq, Xp Amplifications at Xq12, 8q24 11q13	(−) (+)	CGH	(116)
Microdissected human lymph node metastases		13q 9q, 16	(−) (+)	CGH	(117)

Table 4. Multiple Gene Expression changes implicated in prostate cancer metastasis

Gene	Function	Positive or negative effector	*In vitro* (V) or *in vivo* activity (T)	Chromosomal location	Reference(s)
Tazarotene-induced gene 1 (TIG1)	Retinoic acid responder gene	−	T&V(DD)	3q25.32	(118)
Hevin	Extracellular matrix, antiadhesive acidic cysteine-rich glycoprotein	−	T&V (DD)	4q22.1	(119)
NF-κB	Transcription factor	+	V	4q24	(120)
VEGFC	Cytokine(angiogenesis)	+	T	4q34	(121)
Type XXIII Collagen	Transmembrane (type 11)collagen	+	V (rat) (DD)	5	(122)
Endothelin	Cytokine	+	T	6p24	(123)
Src-suppressed C Kinase substrate (SSeCKS/Gravin)	Tumor suppressor	−	V&T	6q24-25.2	(124)
Hepatocyte growth factor	Multiple growth factor-like activities	+	V&T	7q21	(125, 126)
CAT-like	Re-absorption of Ca++	+		7q33-34	(127)
c-erbB2/neu	Cytokine	+	V&T(?)	8p21	(128)
Nkx3.1	Transcription factor	−	T&V	8p21	(129)
Elongin C	Multifunctional	+	T&V	8q21	(130)
Urokinase-type plasminogen activator	Protease	+	T&V	10q22	
Cutaneous fatty acid binding protein	Fatty acid binding	+	V (DD)	8q21.1	(131)
Osteoprotegerin	Cytokine (Osteoblastic)	+	T	8q24	(132)
Prostate stem cell antigen	GPI anchored cell surface antigen	+	T	8q24.2	(133)

(Continued)

Table 4. (Continued)

Gene	Function	Positive or negative effector	*In vitro* (V) or *in vivo* activity (T)	Chromosomal location	Reference(s)
DAB-2 interacting protein (DAB2IP)		−	V	9q33.1-33.3	(134)
Annexin I	Calcium binding adhesion, membrane trafficking, cell signaling	−	T&V	9q12	(135, 136)
Annexin II				15q21	
Parathyroid hormone-related protein (PRHrP)	Peptide hormone	−	T	12p12	(137)
PRHrP receptor	Hormone receptor	+			
C13	Nuclear, glutamine and alpha helix rich	−	T(DD)	13q12-14	(138)
Autocrine motility factor (AMF)	Cytokine	+	V	16q21	(139)
Progastrin-releasing peptide (ProGRP)	Cytokine	+		18q21	(140)
Maspin	Serine protease inhibitor (adhesion to ECM)	−	V&T	18q21.3	(41)
TGF-β family	Cytokine	−	V	19q13	(40)
CLAR1	Proline-rich with SH3 binding domains	+	T (DD)	19q13.3	(141)
Bone morphogenetic proteins (e.g., 6 & 7)	Cytokine (osteoblast differentiation)	+	T	6p24 20q13	(142, 143)
Matrix metalloproteinases	Proteases (tissue and vascular escape)	+	T	Multiple	Reviewed in (144)
Connexins	Intracellular communication	+/−	T	Multiple	Reviewed in (145, 146)

Table 5. Genes expression changes included in the 'Metastasis Signature' (all tumors), (Taken from Ramaswamy et al., (76))

Overexpressed genes	**Chromosomal location**
Elongation factor 4E-like 3 (EIF-4EL3)	2q37.1
Lamin B1 (LMNB1)	5q23-31
Securin (PTTG1)	5q35.1
Heterogeneous nuclear ribonucleoprotein A/B (HNRPAB)	5q35.3
Type 1 collagen a2 (COL1A2)	7q22.1
Small Nuclear Ribonucleoprotein F (SNRPF)	12q23.1
Type 1 collagen a1 (COL1A1)	17q21-22
Deoxyhypusin synthase (DHPS)	19p13.1
Underexpressed genes	
Actin g2 (ACTG2)	2p13.1
RNA binding motif 5 (RBM5)	3p21.3
Myosin light chain kinase (MYLK)	3q21
MHC Class II, DPb1 (HLA-DPB1)	6p21.3
Nuclear Hormone receptor TR3 (NR4A1)	12q13
Myosin heavy chain 11 (MYH11)	16p13.1
Metallothionein 3 (MT3)	16q13
Calponin 1 (CNN1)	19p13.1
Runt- related transcription factor 1 (RINX1)	21q22.3

culture to have a normal karyotype, which could reflect difficulties in culture technology. A more precise estimate can be gained by *comparative genomic hybridization* (68), which employs metastatic tumor DNA labelled with one fluorochrome (test DNA) and normal (male) diploid DNA, preferably but not essentially from the same patient, labelled with a different fluorochrome. The DNA's are denatured and allowed to anneal to form double strands, in the presence of a repetitive unlabelled DNA sample (Cot-1), to eliminate noise. After extensive annealing, the mixture is further hybridised to spreads of normal human karyotypes. Since any gains in the test DNA relative to the normal DNA will be over represented as labelled single strands in the hybridization mixture, the regions on the chromosomes homologous to these sequences will be labelled. By convention these are coloured green. The converse is true for losses in the metastatic cell DNA relative to the normal, when excess labelled, unhybridized normal DNA will be present in the hybridization mixture, and can anneal to its homologous location on the human chromosome (red label). Detailed cytogenetic analysis under ultra-violet illumination, and computer-assisted visualisation identifies the altered chromosomal loci. The technique is more suited to cell line analysis than

extracted tissues, as it requires up to 1 microgram of pure DNA, and data obtained with PC3 and LNCaP metastatic variants confirms the presence of a number of known metastasis associated loci in prostate tumors as summarised in Tables 2 and 3 (e.g., 67).

6.2 Allelic Linkage/Loss of Heterozygosity

Numerous studies of allelic losses and gains in prostate cancer have been published over the last 10 years. Analysis is based on microsatellites in the human genome, which consist of multiple polymorphic repeats of simple di tri or tetra nucleotides. Some of these repeats are associated with pathology, such as the CAG (polyglutamine encoding) repeat in the human androgen receptor. Mostly they lie outwith coding sequences, and inheritance of different unit repeats at the same locus from an individual's mother and father generates a heterozygote. If a section of chromosome or locus (i.e. containing a tumor/metastasis suppressor) is lost from on chromosome relative to normal DNA from the same patient, then loss of heterozygosity (LOH) has occurred (69). High density microsatellite collections are now available for the entire human genome, and have now been supplanted by even more widely and evenly distributed Single Nucleotide Polymorphisms (SNP's) (70). Precise location of genes can be accomplished in this way, but both fragile chromosomal sites and the inherent genomic instability in tumors can generate "noise" in the analysis.

Most LOH studies have focussed on differentiating either normal tissue from tumors or low Gleason grade tumors from high Gleason grade tumors. Whilst the higher Gleason grades have greater *potential* to metastasise, the data do not differentiate advanced, poorly differentiated tumors with multiple genetic changes from truly metastatic lesions. The data are variable, probably as metastatic lesions are both heterogeneous and difficult to obtain. Any conclusions about metastatic genes from LOH analysis are therefore less reliable than with some other technologies (see Table 3). The various gene loci identified by these techniques (Table 3) are also illustrated in Figure 3b.

A different and complementary approach was taken by (71) who exploited the large number of families in their population study for familial tumors. By comparing the 'aggressiveness' of cancers from 513 brothers where the Gleason score of their tumors was known and wide differences were observed, they carried out a traditional linkage analysis with 364 equally spaced polymorphic microsatellites. The results indicated the presence of an additional 3 'markers' of aggressiveness, which could be equated to malignancy at 5q31-33, 7q32 and 19q12 (indicated in blue on Figure 3b).

The sample size was sufficiently large to achieve high statistical significance in the analysis, and at least 2 of these loci correspond to gene loss hotspots.

7. TECHNIQUES TO IDENTIFY DIFFERENTIALLY EXPRESSED GENES

The search for novel metastasis genes in prostate carcinoma is more difficult. As with other studies, in prostate cancer, the lack of effective models precludes decisive results. Many of the results are technology dependent, providing interesting new candidates from *in vitro* studies, which are infrequently confirmed in larger scale studies of human tumor material.

The ability to detect differences between populations of nucleic acids from metastatic and non-metastatic cellular populations has been exploited over many years. However many of the differences are subtle, and technology was unable to resolve these from background until the power of gene amplification was combined with the subtractive hybridization technologies. Also, most of the techniques require rather large starting quantities of RNA, which poses problems in heterogeneous metastatic lesions.

7.1 Subtractive Hybridization

The easiest way to compare two nucleic acid populations is to selectively hybridise them together, to leave an under and over represented population in an unpaired state, where imbalances have occurred. The basic procedure is illustrated in Figure 4, but the enduring problem with a sound methodology has always been the yield of unpaired molecules, which restricted the changes detected to those of great magnitude, or aberrant hybrid formation (72). However, by combination with gene amplification, the technology is able to analyse much smaller differences in expression levels. When combined with use of cDNA micro-arrays (73, 74) and SAGE tagging an even greater sensitivity and identification of multiple new target genes as described later.

7.2 DNA Microarray

Recent advances in mass gene analysis such as printed oligonucleotide gene tissue arrays have resulted in the definition of sets of candidate carcinogenesis and metastasis genes. A meta analysis of the major studies was recently published (75), but like most studies of this type in prostate

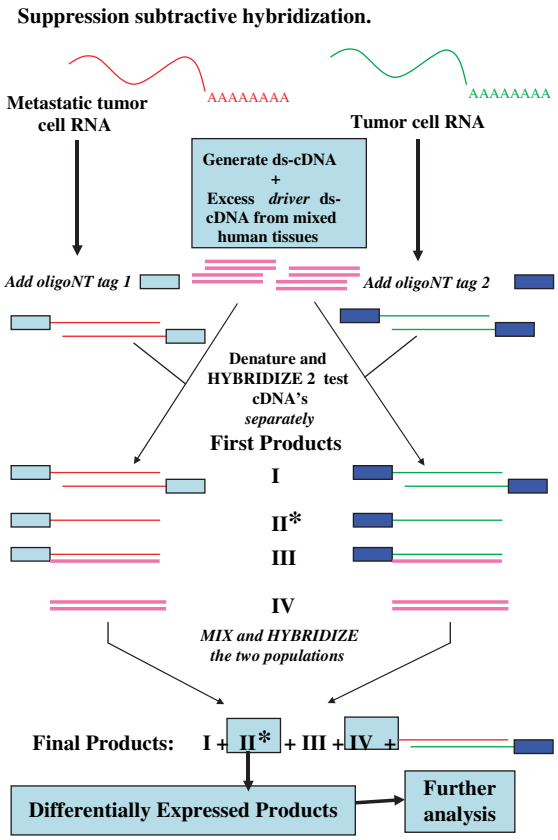

Figure 4. Suppression subtractive hybridization. Comparisons between different nucleic acid populations are compromised by abundant base sequences which can mask significant differences. The suppression subtraction technology, illustrated here for an mRNA comparison, can be extended to DNA by initiating the comparison after the RT step. The suppression step with excess ''driver' cDNA reduces background from abundant mRNA's. Population II (indicated by *) represents the unique species from the subtraction, to be used either as templates for PCR amplification (employing the tag for example) or for direct labelling and hybridization to microarrays. The final analysis step can be by either comparative genomic hybridization to metaphase chromosomal spreads or to DNA microarrays (the 5' oligonucleotide tagging (OligoNT) step is not necessary for microarray).

cancer, obtaining sufficient material from genuine metastatic lesions to carry our the analysis remains a problem. However in the study by Dhanasekaran et al. (65) 20 metastatic samples were included in the analysis, with an equivalent number of benign (19) and localised samples (14). Even allowing for the heterogeneity of prostate tumors, this population of metastases can

provide clues as to the key over-expression products, which could be linked to genetic deletions and amplifications. Aside from some previously determined over- and under-expression products such as e-cadherin (−), PTEN (−) fatty acid synthetase (+) and c-myc (+) the major products which emerged as diagnostic aids were hepsin (+), maspin (−) and AMACR (+), although their mechanistic relevance to the metastatic process remains questionable.

For a definitive analysis of genes over-expressed in metastasis, the best measurement has currently been obtained from 64 primary and 12 metastatic adenocarcinomas originating from prostate, lung, colon, breast, ovary and uterus (76). The initial screen produced a set of 128 genes, which could distinguish the metastatic lesions from primary tumors. However, some primary tumors also showed altered expression of genes from the distinguishing set. The authors raised the possibility that these organ-confined tumors already contained cells pre-programmed to metastasise, particularly with lung tumors. This could equally apply in those "difficult" prostate tumors with a Gleason score of 5–7 where prognosis is a major diagnostic problem. Further refinement of the data set resulted in a minimal signature of genes over and under-expressed in metastases. Without further confirmation by RT-PCR or northern blotting, the minimal set of 17 genes was applied to sets of test tumors, including 21 prostate tumors, where the prediction had a p value of 0.022 (77). The genes included in the 'metastasis signature' are listed in Table 5 and mapped on to the human karyotype in Figure 3d. The predictive power applied to a range of tumors, indicating that the genes whose expression must be altered in order for a tumor to metastasise is probably a basic biological function, independent of the tissue of origin, although tissue-specific functions do probably exist.

Finally, the power of microarray analysis may not be exploited to its fullest extent, or in an extreme case, be providing misleading data. Most analyses reduce the differential expression to a ratio, relative to 'normal' tissues. There are good statistical reasons to be cautious with this approach, and the distinct possibility remains that these analyses are detecting over-expression epiphenomena; a result of signal amplification from a critical upstream event, which may be more subtle (78).

7.3 Differential Display

One of the earliest methods of comparative gene expression, differential display (DD) has been used to analyse differences in gene expression between normal and tumor cell from prostate (79), but the required amounts of RNA for the analysis are relatively large, which precludes use with small

metastatic lesions. By selection and cloning of individual products, the DD technology can isolate individual genes based on different sequence and biological criteria, but the procedure can be time consuming. To accelerate gene discovery, it can be combined with cDNA microarrays (80) to reveal multiple expression alterations between metastatic and non-metastatic cell lines. These candidates remain to be confirmed on tissues however. A number of candidate genes have emerged from DD analysis. Some of these are listed in Table 4 (indicated by DD).

7.4 Serial Analysis of Gene Expression (SAGE)

This technology was devised to overcome the laborious nature of differential display, by amplifying differentially expressed sequence tags of 10 base pairs as concatamers with defined ends. The small sequence tags are finally used to screen sequence databases to identify specific products, whose expression changes are confirmed by other technologies in the target tissue (81). It is considerably faster than expressed sequence tag (EST) analysis, from which it is derived, and with the human genome map now complete (April 2003) final analyses and gene identification will be more rapid.

With prostate cancer, SAGE analysis has identified a number of expression changes such as E2F4 and Daxx (82) from a total of 156 detected changes. Links to metastasis have still to be confirmed. In a separate study genes implicated in the evasion of cellular senescence in prostate cancer were analysed by SAGE. In this case 273 changes were observed, which could be related to both phenotype and senescent stage. The data has to be extended to tissues however (83).

In summary therefore, the listing of 'metastasis associated' gene expression changes in Table 4 is unlikely to be complete, or *universally* applicable, given the technique (and clinical material) dependency of the analyses carried out. Most likely the sheer number of changes observed reflects gross perturbations in gene expression required by the metastatic cell to survive in its new extra-prostatic environment. The most common genes, whose expression changes are recorded in Table 4, have been mapped on to the human genome in Figure 3c.

7.5 Proteomics: Analysis of Gene Products in Metastasis

All of the previously discussed genetic changes will provide either a chromosomal or an expression fingerprint for metastatic cancer, although it

is obvious from the preceding that considerable refinement is still required. As far as mechanistic genetics are concerned, translation of the DNA and RNA studies into altered protein levels is necessary. The main aims of the 'post-genomic'era are to develop the proteome, and its application to the genetics of metastasis in prostate cancer are still relatively primitive, compared to the nucleic acid studies. Again however, the quality of the results obtained will be determined by the strength of the biological systems. To permit proteomic analysis, new technologies for the precise analysis of the many protein forms within cells have been developed (see review by Nelson et al. (84)). The sheer complexity of protein expression patterns can be simply explained as follows: In the human genome there are about 40,000 genes. Many of these produce multiply spliced mRNA, which results in translation into different polypeptide chains. These polypeptides are further modified by proteolysis, glycosylation, phosphorylation etc, to produce protein with often radically different biological activities.

The application of proteomics to the study of prostate cancer metastasis can be divided into several enabling technologies as follows:

7.5.1 Analysis of the Proteome of Extracted Prostatic Tissues and Cells

To assist in the analysis of the total proteome, databases from multiple prostate tumors are being assembled (e.g., Nelson et al. (85)). To achieve cellular homogeneity, microdissection has to be carried out. However the technique does not always produce reliable results on traditional formalin fixed tissue, and frozen or ethanol fixed tissues must be used. A minimum of 10^5 cells is required for the 2D polyacylamide gel electrophoretic analysis, followed by spot picking and conventional protein mapping by mass spectrometry, which could restrict its use in multiple metastatic lesions. To date there is no information on the proteome of bone metastasis for example, although the difficulty in applying the technology was recently confirmed by Ahram et al. (86), who compared 12 matched normal prostate samples with corresponding high grade tumors (presumably with metastatic potential). The results confirmed the heterogeneity of prostate cancers at this level of analysis, since despite detecting forty changes in protein constitution, none were conserved between all of the tumors. No really new candidate metastasis associated genes were identified, although genes previously expressed in tumors such as lactate dehydrogenase, laminin receptor and tropomyosin-β were upregulated.

SELDI analysis, which has been developed for the analysis of small volumes of serum (see below), can equally be applied to the analysis of the

protein complement and differences between microdissected cell populations (87). The problem remains the assignment of the peaks from the MS analysis to specific protein for which complex algorithms are only now being developed (88, 89)

7.5.2 Tissue and Antibody 'Chips' to Facilitate Large Scale Marker Screening

The external surface presented by metastatic tumors to its environment is important for interaction with other cell types, matrix and of course the immune system. Many studies have analysed the distribution of individual cell surface proteins on prostate cancer cells, with a resulting heterogeneity frequently observed. Liu (90) adopted a more general approach, using a forerunner of commercially available antibody arrays to produce 'macroarrays' each containing 16 immobilised antibodies to screen the expression of 119 different CD antigens in the common prostate cancer cell lines. Unsurprisingly, all were different and also displayed heterogeneity of staining intensity *within* populations of the same cell culture type, for example for CD44 antigen. While the paper speculated about this heterogeneity, it could simply be a result of genetic instability in the established tumor cell cultures. Similar analysis of metastatic lesions should produce important data about immune targeting, for example, although the disaggregation methods to be used will have to be carefully controlled to avoid antigen degradation.

There are now commercial antibody microarrays, to enable the researcher to probe multiple components of intracellular signalling pathways, activated in cancer and apoptosis, for example.

7.5.3 Analysis of Serum Markers of Metastasis

A serum protein to enable early and error free detection of metastatic prostate cancer is the ultimate goal of many marker studies (see review by Bok and Small, (91)). There already exist 2 examples of genes detected by this method. Both have been known for more than 10 years! The first, and still a reliable marker of metastatic disease was prostatic acid phosphatase (92). A better marker, of course is PSA, whose serum levels have been exploited extensively (93). The major drawback in using these two secreted proteins is their lack of specificity for metastatic disease. Elevated levels can be the result of a number of non-malignant conditions, and a search for new markers using proteomics has been under way for several years. The problem here is of course the source of material. Variously, blood (serum)

ejaculate and prostatic fluid expressed after massage have been used as a source of material. The problem of course remains the complex nature of all of these fluids. Prostate cancer cells have been detected in serum by a variety of means, including RT-PCR for tumor-specific markers such as PSA and PSMA (94). However, the protein content to be analysed is sufficiently complex to confound visual or even computer assisted analysis. The increasing database of known proteins should facilitate such analyses.

In a recent antibody microarray study (95), a comparison of the protein profiles in serum from 33 prostate cancer and 20 normal serum samples using a 183 antibody microarray detected significant differences between the two populations in the expression of five proteins: von Willebrand factor, immunoglobulins G and M, alpha-1 antichymotrypsin, and villin. These could form the basis of new tests for early metastasis detection.

The choice of technology for this type of analysis (96) lies between the conventional 2D gel separation followed by mass-spectrometry assisted sequencing and confirmation of protein identity (97). A simpler technique, now employed routinely for serum analysis is direct separation of protein components by Surface Enhanced Laser Desorption Ionisation (SELDI)-Time of Flight Mass Spectroscopy (TOF-MS) (98). The Ciphergen SELDI has been used to advantage to separate unknown serum proteins to provide a further fingerprint, by making use of the multiple affinity spots on the proteinchip arrays. These act as successive protein fractionation steps to remove abundant serum proteins such as albumin and gamma globulin, to allow more detailed analysis of minor species (normally masked in 2D gel analysis) which could provide the key to better and earlier diagnosis of metastasis, for example PSMA (99). By applying ‘reverse genetics’ the peptides recovered from the time of flight mass spectrometer can be used to isolate the genes involved. It is interesting to speculate, given the diversity of genes and loci in Tables 2–5 and in Figure 3, whether alternative methodology simply provides a further layer of complexity.

7.6 In Silico Searching: Genes at Your Fingertips

The availability of public databases associated with or produced as a result of the human genome mapping program, allows basic research results to be put into a wider context. For example, most enzyme activity has down and upstream consequences. Therefore perturbation of one stage of a pathway has measurable effects on the connected steps in signal transduction or metabolism (for example). However, the situation is complicated by salvage, branched and alternative pathways, which are also activated when a particular reaction is inhibited or over-stimulated. The capacity to measure

all of these effects is beyond the capacity of the human intellect, and computer algorithms come into their own.

For prostate, specific gene expression and structural changes have been collected into a series of such databases: the Prostate Expression database (85) at http://www.pedb.org. This allows rapid screening for the normal and malignant expression patters of more than 20,000 human genes, and is linked to a similar murine database for use with animal models. Such databases have been used to mine for prostate-specific products, and by combination with specific libraries from different cell and tissue types to electronically derive candidate genes for further study as markers of malignancy (84, 100). This type of analysis can even be carried out completely electronically using the Binary Indexing Search Algorithm (101). However the approach does have some limitations, and of course all results must be confirmed experimentally. The technique has identified two new cancer associated expression differences, in cysteine-rich secretory protein 3 (CRISP3, upregulated by more than 50 fold) and de-adenylating nuclease (DAN, downregulated by more than 80%). Linkage to *metastatic* disease was not reported however.

7.7 Metastasis Genes: How Many Activation Events Are Required or . . . Making Sense of the Data

The information which we are being deluged with as a result of both genome wide scans, analysis of the total proteome and microarray for both DNA and mRNA expression changes seems to suggest that an almost infinite number of changes in gene structure and expression are required for the development of prostate cancer metastasis. Recent estimates of around ten independent events (3) seem extremely wide of the mark if the number of gene loci in Figure 3 a–d are summed. What should be considered are the critical genetic changes. Many of the changes above, although seemingly important in prostate, could easily represent epi-phenomena, or downstream reactions to the changes in environment at the metastatic site. The key changes included in Figure 1 are often represented in prostate, allowing escape, survival and re-establishment of the tumor cells outside of the prostatic capsule. Some are clearly bone specific, allowing the prostate tumor cells to proliferate and/or survive.

The most telling results are those from the general survey of Ramaswamy et al. (76) which does reveal a general ‘fingerprint’ of metastasis, independent of tumor type. Are these gene expression changes fundamental? More probably not; given the nature of the genes, they probably represent further downstream effects.

Perhaps a significant feature of the genes in Table 5 is that at least some of the over-expressed products are normally expressed in *stromal* cells, while four of the downregulated genes are *smooth muscle* and two are *haematopoetic* cell genes. Does this expression pattern represent at least part of the epithelial: mesenchymal transition which is a frequent property of malignant tumors (see review by Thiery, 63). Two of the major mediators of this transition, TGF-β and HGF are both upregulated genes in prostate cancers, and the intracellular responses to such signalling in terms of apoptosis induction are short-circuited in metastatic lesions. Indeed one of the most reliable stromal markers: vimentin: is frequently over-expressed in malignant prostate tumors and can provide a good indication of metastatic potential in the primary tumor (102). A similar situation is found for cyclo-oxygenase 2 (COX2), which is expressed in prostate stromal cells in the premalignant state but over-expressed in the epithelium in the malignant state (103, 104). There are numerous other examples in prostate to suggest that the malignant cell has compensated for the lack of stromal contribution by establishing autocrine signalling loops to replace the paracrine signalling in the normal state. The signals probably have very little to do with growth, but rather cellular survival and motility. There also exists a clear role for reactive stroma, and bone stroma for the survival of the metastatic prostatic carcinoma cells (105, 106). Several studies, for example Macintosh et al. (107) have suggested that the changes in the stroma may extend from expression reprogramming to permanent genetic changes.

8. CONCLUSIONS

The data summarised above are neither comprehensive nor conclusive. There are many more anecdotal reports of phenotypic changes in metastatic prostate cancers not included, and more candidate genes are being identified almost monthly. However all of these are the result of genetic changes, and the various levels of control of gene expression discussed earlier. Some of these genetic changes are transient, some are of fundamental importance, while others may be unimportant to the development of the metastasis, but remain as reliable markers of extra-prostatic disease. As the knowledge base increases, it is likely that “metastatic prostate cancer” will be subdivided into a number of tumor types with a sound scientific basis, and an accurate set of prognostic indicators.

A meta-analysis of Figures 3a–d serves to underline this conclusion. On the surface there is little in common between the multiple loci identified by different technologies, although losses at 8p, 10q and 18q are consistently

observed, being detected by all 4 technical approaches. A number of loci are identified by 2 approaches, but given that there is no requirement for downregulation of expression to be reflected in gene loss (although the gene loss is related to expression changes), whereas gene amplification can be directly related to overexpression (for example amplification/overexpression of the c-myc oncogene on chromosome 8q), this type of analysis is probably too premature, until the gene loss/amplification studies are further refined to more precise locations. This can readily be achieved by further exploitation of microarray technology (108).

The biggest danger for the future is to confuse a genetic change with diagnostic potential, with a fundamental change, which could be exploited in therapy. The ideal therapeutic target should be tumor restricted, and essential for the survival of the tumor in the metastatic site. The same is true for immunological targets. Prostate metastases have a capacity both to hide from the immune system, and to vary protein/antigen production, either as a population or a fraction of a tumor mass. By attacking the wrong targets, we would simply be producing new classes of recurrent tumors, as observed in the changes within androgen receptor signalling pathways (both androgen and growth factor mediated) after use of anti-androgen therapies. The current status of prostate cancer genetics is akin to an unedited electronic manuscript: most of the information is out there, some of it is in the wrong place (although some *is* misleading because of imperfect biology), but it must be referenced, annotated and assembled into a coherent whole for the benefit of more than 20,000 new patients diagnosed every year with CaP in the UK.

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