

TARGETING ACTIN REMODELING PROFILES FOR THE DETECTION AND MANAGEMENT OF UROTHELIAL CANCERS - A PERSPECTIVE FOR BLADDER CANCER RESEARCH

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1. ABSTRACT

The actin cytoskeleton and numerous proteins associated with its regulation and function constitute over 25% of total proteins in the cell. Growing evidence from this laboratory and others shows that alterations of actin polymerization, or actin remodeling, plays a pivotal role in regulating the morphologic and phenotypic events of a malignant cell. The actin remodeling is the result of activation of oncogenic actin signaling pathways (e.g., Ras and Src), or inactivation of several important actin-binding proteins that have tumor suppressive functions (e.g., Gelsolin, E-Cadherin, etc.). Recently distinctive protein expression patterns of some of these genes in bladder cancer carcinogenic and progressive processes have been observed. Specific actin-pathway antagonists that have growth inhibitory effect on transformed cells, but not normal cells, have been developed. Our overall hypothesis is that actin alterations are progressive and that distinctive actin remodeling profiles are associated with different stages of cancer development and progression. These patterns can be used as markers for cancer early detection and prognostic indication. On the other hand, detection of specific types of actin-signaling pathway alterations also enables a targeted preventive or therapeutic intervention with specific actin signaling pathway blockers, thereby providing an actin-based paradigm for individualized monitoring and intervention of human bladder cancer.

2. INTRODUCTION

Although much progress has been made in recent years in identifying molecular events that lead to the development of cancer, the exact mechanisms underlying the evolvement of malignant phenotypes remain poorly understood. For instance, it is not clear what exact biochemical events causing cell shape changes seen in a malignant cell are. It has generally been assumed that alterations of cytoskeletal proteins such as microfilament actin are involved, but how they occur is still not exactly clear. Understanding such mechanisms may have an impact in developing novel strategies for cancer management, including detection, monitoring, and targeted intervention.

Actin was first identified in non-muscle cells in only about four decades ago, and at about the same time, it was found that actin filaments were disrupted in the malignant transformed cells (1). The actin network is a rather complex, yet important structural and functional system of all eukaryotic cells (2). Recently tremendous progress has been made in understanding the molecular mechanisms of actin regulation and remodeling. Extensive studies have demonstrated that actin filaments are regulated by actin signaling proteins (ASPs) that are components of important oncogenic signal transduction pathways, the most notable one is the small GTPase of Ras superfamily proteins Rac, Rho, and Cdc42 (3,4). On the other hand, a large number (over a hundred) of actin

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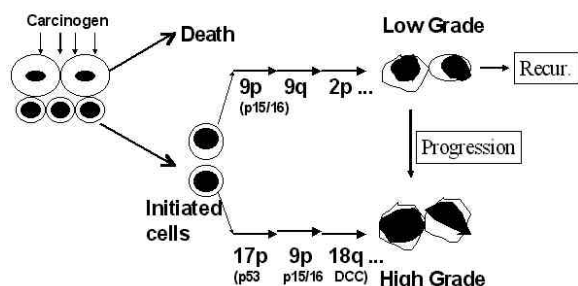


Figure 1. Molecular progression pathways for bladder cancer.

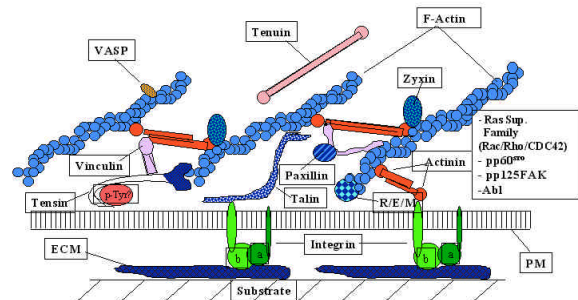


Figure 2. Actin involvement in focal adhesion (modified from Ref (5)).

binding proteins (ABPs) have been cloned, and the list keeps growing (6,7). Many of these proteins are invariably involved in malignant transformation process (8,9,10). The purpose of this review is to provide a brief overview of our current understanding of how the alteration of actin network might be involved in malignant transformation and in turn, discuss the potential for developing an actin-based bladder cancer detection and management schema.

3. THE NEED FOR DEVELOPING BIOMARKERS FOR BLADDER CANCER MANAGEMENT

Bladder cancer, like other malignant tumors, develops through multiple genetic and epigenetic alterations that lead to the altered growth, differentiation, and apoptosis control (11,12). It provides a useful model system to examine the actin-based detection and management schema because it has well-defined disease progression stages (from premalignant dysplasia, to low-grade, and to high-grade lesions) and relatively easy access to the entire organ through urine cytological and cystoscopic examinations (13). Recent evidences suggested that bladder cancer might follow two development pathways (Figure 1). One pathway begins with chromosome 9 (LOH of 9p and 9q) alterations (14) that results in the development of low-grade tumor, and the other pathway involves an alteration of p53 gene which lead to the development of a high-grade tumor. Although less than one-third of low-grade tumors will progress to high-grade form, over two-thirds will recur periodically. The number of deaths from low grade bladder cancer that progresses is about equal to the number who die from bladder cancer that was originally invasive. The current

regime of periodic cystoscope coupled with conventional urine cytological examination is neither cheap, nor effective. This approach overall has rather low accuracy in detecting tumors, specifically the low-grade forms, neither it can predict the tumor behavior nor the therapeutic response.

Although numerous biochemical and molecular markers have been developed as possible adjunct tests for urine cytomorphological analysis, many these tests are either too expensive, tedious to perform, or suffer low specificity (15). The p53 or Rb by simple immunohistochemical methods may be useful as prognostic indicators, however, such tests require resected tissue samples and the results are far from ideal. Therefore, more accurate early detection markers and prognostic indicators are urgently needed, and expression profiles of actin remodeling genes may assume such roles.

4. ACTIN AND ACTIN BINDING PROTEINS

Actin is a 43Kd protein that is highly conserved between species (2). There are three main actin isotypes (alpha, beta and gamma) which show >90% amino-acid (aa) homology between isotypes and >98% homology within members of a particular isotypic group. Actin exists in cells as two basic forms: globular, nonpolymerized G-actin and polymerized double-helical actin filament (F-actin) (16). The G-actin readily polymerizes under physiological conditions to form F-actin with the concomitant hydrolysis of ATP, and the actin polymerization and depolymerization processes are carefully regulated by a number of important signal transduction events (3,4,17), as we will discuss more below.

F- and G-actin interact with a multitude ABPs (2), which can be divided into two general categories. The first category is actin bundling and crosslinking proteins. These proteins help maintain the cell viscosity and cell shape, and at the same time link the actin cytoskeleton to the plasma membrane by interacting with both actin and membrane proteins, such as integrins and the Fc receptor, and may help direct signals to the actin cytoskeleton. Other members of ABPs in this category involve in the interaction of F-actin with myosin (e.g., tropomyosin), therefore they are related to cell motility and contraction (18). Examples of the ABPs in this category are alpha-actinin, E-cadherin, filamin, vinculin, talin, etc (2,19). The second category of ABPs is involved in regulating actin polymerization and assembly process (20). Some ABPs preferentially binds to the F-actin, these include two main families of barbed-end capping proteins: gelsolin/villin family members, which either cap only (like CapG) or both sever and cap filaments (like gelsolin, villin and adseverin) (6,21). Others mainly bind to G-actin (Thymosin, Profilin, Cofilin, etc). Proteins that bind to G-actin generally cause G-actin sequestration, thereby inhibit actin depolymerization, whereas proteins that cap the F-actin prevent polymerization. However, Gelsolin family proteins can also accelerate nucleation, therefore increase the rate of polymerization. Figure 2 presents a model of how actin is involved in focal adhesions.

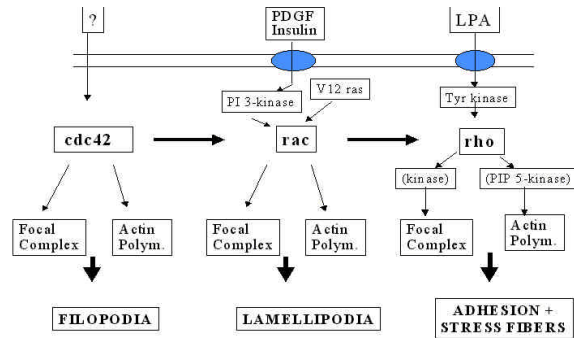


Figure 3. Actin signaling (adapted from Ref. (4)).

5. REGULATION OF ACTIN ASSEMBLY DYNAMICS AND ACTIN SIGNALING

The actin polymerization/depolymerization is a rather complex process with several carefully controlled steps. G-actin will spontaneously form trimeric (or tetrameric) nuclei at a very slow rate and polymerize to form filamentous actin (F-actin), processes favored as actin concentration and ionic strength increase (22). The F-actin is a double-helical, polarized structure with plus and minus ends. Actin polymerization is controlled by G-actin binding proteins and by F-actin-capping proteins. The most abundant of the former is thymosin, a 5 kDa protein, which binds to ADP G-actin thereby "sequestering" it and preventing its addition to F-actin (23). Profilin, another actin-binding protein that has the same activity as thymosin, but that can also promotes monomer incorporation into filaments by stimulating the exchange of bound ADP for ATP (20). In certain situations, profilin can associate with the plasma membrane phosphatidylinositides as a consequence of the action of an extracellular signal.

Gelsolin is an F-actin binding protein that clips filaments and then remains bound to the plus end, serving as a cap that blocks further filament growth and prevents depolymerization (20). Gelsolin's activity is stimulated upon the rise in intracellular Ca^{2+} , thus, it can be regulated by extracellular signals. Like profilin, gelsolin binds phosphatidylinositides in the plasma membrane, which inhibit its activity allowing F-actin growth (20).

Individual actin filaments are assembled into two general types of structures, called actin bundles and actin networks. In bundles, the filaments are cross-linked in compact parallel arrays. In networks, the filaments are loosely cross-linked in orthogonal arrays. The formation of these structures is mediated by various F-actin-binding proteins, which contain at least two actin-binding domains. Actin-bundling proteins are small, rigid proteins that force a close alignment of parallel filaments. In contrast, actin-networking proteins tend to be large flexible molecules.

Actin signaling is a process of transferring signals from extracellular ligand/receptor complexes to induce phenotypic responses of a cell such as cell shape changes and cell movements. These phenotypic responses are brought by altering the actin assembly dynamics that are regulated by several oncogenic signal transduction pathways (3,4,24,25). These pathways are overlapping

functionally yet mechanistically distinct. The key element in controlling actin dynamic is the Ras-family small GTPase Rac, Rho, and Cdc42 (3,4). Figure 3 shows how each of these GTPase induces different cell shape changes in response to various extracellular signals: Cdc42 induces filopodia, Rac induces lamellipodia in response to PDGF stimulation, whereas Rho induces stress fiber formation in response to LPA. The other two major regulators of actin dynamics are Src and Bcr-Abl family proteins, both involve in focal adhesion formation and their effect on actin appear to be mediated by their tyrosine kinase activities. The Src-induced actin dynamic change is mediated by tyrosine kinase activity of its SH3 domain, apparently through the binding of SH3 domain to PI-3 kinase and activation of the downstream Akt (25). The Bcr-Abl oncogene, which causes chronic myelogenous leukemia, contains an actin binding domain on the c-terminal end of Abl (26). Studies have shown that mutations of this actin binding domain reduce the oncogenicity of this oncogene (27). The Bcr-Abl oncogene activity causes adhesion defects by its SH3-like tyrosine kinase activities directly on F-actin itself and actin binding proteins such as paxillin (24).

6. RELATIONSHIP OF ACTIN REMODELING WITH TRANSFORMATION

At about the same time when actin was discovered in non-muscle cells, Weber and his colleagues demonstrated that, upon malignant transformation of NIH3T3 cells by SV40, actin fibers disassemble, and reassemble when the transformed cells revert to normal cells by a spontaneous mutation (1). Years later it was found that the altered actin assembly appears to be mediated by the oncogenic effects of the mutant K-Ras and H-Ras oncogenes (28). However, it was unclear whether such alteration contributes to malignant transformation or is only a byproduct of cellular transformation. Recently, compiled evidence began to reveal that actin remodeling plays a pivotal role in carcinogenic process. These findings are summarized below:

1. We observed that cytoplasmic actin depolymerization, as measured by a decreased F/G-actin ratio (increased G-actin with concomitantly decreased F-actin), is a marker of cellular dedifferentiation (29), and early transformation (30), and therefore can be used as a marker for early detection of cancer (31). A careful mapping study showed that cellular G-actin level not only increased in most tumor areas, and in also more than half of the distant fields of tumor bearing bladder (32). A subsequent study showed that an increased G-actin in the field could be a marker to monitor chemopreventive effect and tumor recurrence (33).
2. The nuclear actin is also altered in malignant cells and such alteration might be one of the important underlying mechanisms for the overall genetic instability, the hallmark of cancer cells (34).
3. Actin itself is a major morphological effector in apoptotic process, and stimulating actin polymerization enhances apoptosis induction in both chemotherapeutic sensitive and resistant malignant cells (35, 36).
4. Early studies have demonstrated that increased actin turnover rate and disorganization in individuals genetically

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Actin Associate Molecules Implicated in malignant transformation

- Oncogene signal transduction pathways
 - Ras family (GTPase):
 - Rho (stress fibers)
 - Rac (lamellipodia)
 - Cdc42 (filopodia)
 - Src family (tyrosine kinase)*
 - FAK*
 - Tumor Suppressor
 - Gelsolin*
 - Tropomyosin/merlin
 - Alpha-actinin*
 - E-cadherin
 - Beta-Catenin
 - Vinculin
 - Fodrin*
- * Relate to integrin signaling *Implicated in apoptosis

Figure 4. Actin associated molecules implicated in malignant transformation.

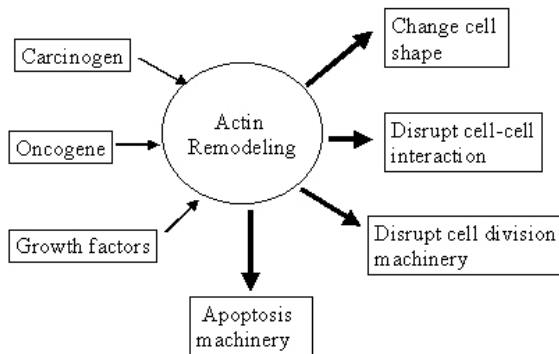


Figure 5. Actin remodeling in transformation

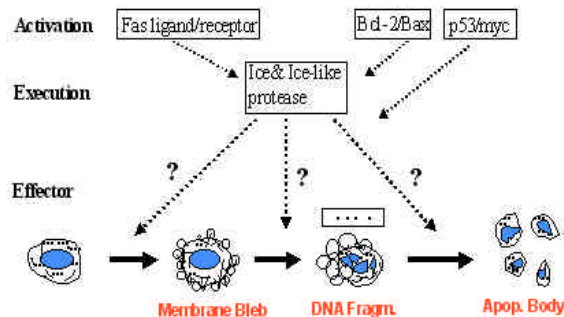


Figure 6. Apoptotic machinery.

predisposed to cancer indicate that alteration of cytosolic actin may be an early event in tumorigenesis (37), and a mutated β -actin gene contributes to the neoplastic phenotype of immortalized human fibroblasts (38).

5. Pienta and others have shown that actin forms a physical connection between the nucleus and the cell periphery which is altered by transformation (39, 40). Such connection may involve in several major oncogenic signal transduction pathways.

6. As shown in Figure 4, at least three oncogenic signal transduction pathways are involved in actin signaling. These pathways are Ras superfamily GTPase proteins Rac/Rho/Cdc42 (4), Src (25), and Bcr-Abl (26). A mutation of F-actin binding domain of C-Abl causes a reduced ability

to transform Rat-1 fibroblast cells (27). Recently a landmark study using gene chip technology identified Rhoc as a major player in metastatic behavior of malignant melanoma (41).

7. Recent evidence shows that p53 binds to F-actin directly and such binding has functional significance (42).

8. Also shown in Figure 4, many ABPs show tumor suppressive functions. Examples of these ABPs include Gelsolin (9), tropomyosin (10), vinculin (8), etc., and the list keeps growing. Interestingly, actin itself and some of the ABPs are also targets of caspase activity (43,44,45,46), the intermediate of apoptosis executor.

9. Similar changes of actin polymerization status as we seen in bladder and breast cancers have also been observed in other cancer types (prostate, lung, and endometrium) that have been examined by various investigators (47,48,49,50).

Taken together, a simplified model for our current understanding of how actin remodeling is involved in carcinogenic process is depicted in Figure 5. Overall, in a given cell, alteration of actin remodeling appears to be a generalized effector event for many oncogenic signal transduction and growth factor stimulation activities (51,52,53,54,55). The altered actin in turn provides the basis for altered cell shape. At the same time it may also contribute functionally to the disrupted cell division and cell-cell interaction (through integrin) mechanisms. Add to this picture is another whole dimension of cell death, or apoptosis machinery that actin is involved in, as discussed in following section.

7. RELATIONSHIP OF ACTIN REMODELING WITH APOPTOTIC PROCESS

Apoptosis is a regulated process while the molecular machinery is conserved through evolution (56). Understanding molecular mechanisms of apoptosis is important in developing new cancer intervention strategies. As shown in Figure 6, at the morphological level, the apoptotic process is characterized by sequential changes that usually begin with membrane blebbing, followed by DNA fragmentation, and finally apoptotic body formation (57).

At the molecular level, this process can be arbitrary divided into three phases: upstream activation phase, intermediate “executioner” phase, and down stream effector phase (56). The upstream pathways include activation of Bax over Bcl-2, binding of Fas receptor by Fas-ligand, and co-activation of p53 and myc. The intermediate mediators, or so called “executioner”, are members of the human Interleukin-1 Converting (ICE) protease superfamily, or caspase family. In the effector phase, the apoptotic mediators cleave a number of structure and enzymatic proteins that eventually lead to the typical morphological changes seen in apoptotic cells. Although many substrates have been identified to date, it is far from clear which substrate plays a major role as a morphologic effector in such process (56).

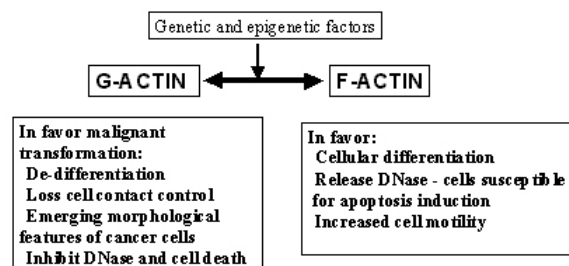


Figure 7. A hypothetical model of how actin function as a switch in transformation and cell death process.

Since apoptosis tends to be morphologically similar among different cells, it has been speculated that the most important substrates should be ubiquitous and evolutionary conserved. Members of actin network proteins including actin itself and ABPs, are idea candidates in this setting. Indeed, recently several studies observed that actin (43,44) or actin remodeling proteins, fodrin (45) and gelsolin (46), are substrates of the caspases activity. *In vitro* studies showed that cleavage of G-actin by caspases yielded small actin fragments (from 45 KDa to 41, 30, or 15 KDa) that were no longer able to polymerize to F-actin, or to inhibit DNase I (43). A further study from Chen demonstrated a direct involvement of this cleaved actin in chemotherapeutic agent induced apoptosis in solid ovarian cancer cells (58). The cleavage of actin or actin-modulating proteins by caspases presumably alters the homeostasis of the actin polymerization status. This may in turn induce typical morphological changes seen in apoptotic cells.

However, a more recent study from Song failed to observe the actin cleavage *in vivo* during apoptosis in a number of cell models including a Burkitt's lymphoma cell line, a lymphocytic leukemia cell line U937, and HeLa cells (59). The findings of this later study questioned the hypothesis that cleavage of G-actin is a central and generalized theme in the apoptotic process. Our hypothesis is that alteration of actin polymerization process probably represents a more generalized phenomenon. This hypothesis is based on the observations made in our recent study using Camptothecin induced HL-60 apoptosis as a model (35). Using this model system, coupled with a quantitative monitoring of actin change with specific morphologic events, we observed a unique actin alteration pattern in apoptotic process. In contrast to transformation process, the apoptotic process was characterized by actin polymerization, at least initially, and the apoptotic morphologic events seem to be regulated by actin polymerization changes. Furthermore, stimulation of actin polymerization by Jasplakinolide (Jas) induced apoptosis directly, whereas inhibition of actin polymerization by agents Cytochalasin B (CB) or E (CE) inhibited CPT induced apoptosis. The Jasplakinolide induced apoptosis is mediated by the DNase I activity. Furthermore, we also demonstrated that the pattern of actin polymerization change correlates with specific morphological events of apoptosis (membrane bleb formation, DNA fragmentation, and apoptotic body formation).

Similar findings have been reported by others using different model systems as well (60,61,62). These studies together showed that the regulation of actin polymerization is an important apoptotic morphological effector, whereas the alterations of the actin polymerization status by chemicals have profound effects not only on altering the morphology of apoptotic cells, but also on apoptosis induction in various cell types.

In summary, the distinctive patterns of actin remodeling in transformation and apoptotic process prompts an over-simplified hypothetical model, as shown in Figure 7. Generally, in response to various genetic or epigenetic stimulators, the actin remodeling may function as a phenotypical switch for apoptosis and transformation, depending on the cell type and differentiation stage. Shifting of actin to polymerized form indicates that the cell is more differentiated, and such cell is more prone to apoptosis induction. In contrast, shifting to G-actin indicates a less differentiated and even transformed status of a cell, which is less susceptible to apoptosis induction.

8. ACTIN REMODELING PATHWAYS AS NOVEL TARGETS FOR CHEMOTHERAPEUTIC AND CHEMOPREVENTIVE DRUG DEVELOPMENTS

Because cytoskeletal proteins play such critical roles in the regulation of cell structure and function, including cell growth, differentiation, and apoptosis, it is thus without surprise to see that they have been the focus of new chemotherapeutic drug development in recent years (for review see Ref (63)). Drugs targeting microtubules, such as Taxol, have been used successfully in the treatment of a number of cancer types. Although there has been no actin targeting drug available at this time, active studies are undergoing in mainly two major areas. The first is to develop chemicals that regulate actin polymerization directly and the second is to target specific actin signaling pathways, mainly the ras pathway associated small GTPase moleculars. Examples of actin polymerization chemicals include Cytochalasins (B, D, and E), the actin depolymerization agent, and Jasplakinolide (Jas), a potent actin over-polymerization agent (64,65,66). Jas has been shown to have significant growth inhibitory effect on breast and prostate cancer cells *in vitro* (65). It is a unique agent in that it stimulates actin polymerization on one hand, but disrupts F-actin fibers, presumably the insoluble component of actin, by blocking the focal adhesion kinase (FAK) signal pathway, on the other (66).

Although it is still in its infancy, the search for actin signaling pathway specific blockers targeting Ras family small GTPases is a promising area of research. A number of peptides have been developed, some of which showed selective effects in inhibiting the tumor cell growth, but not the untransformed cells, at least under the *in vitro* situation. An example of one of such peptides, ACK-42 is a CDC-42 specific inhibitor developed by Marauta, *et al* (67). It is a 42-amino acid fragment of the Tyr-kinase ACK-1 that binds only CDC42 in the GTP-bound form, and it would selectively block the interaction

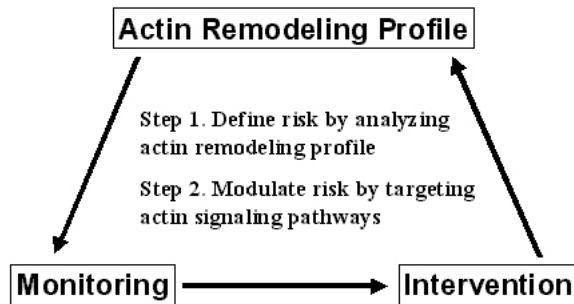


Figure 8. Actin based bladder cancer detection and management schema.

of CDC42 with any of its effectors. Maraut, *et al* has found that over expression of ACK42 suppresses Ras transformation, and a cell-permeable derivative of ACK42 called WR-ACK42 selectively blocks the growth of Ras transformants without any effect on the parental normal cells (67). The development of such arsenal, if prove to be successful, is highly significant in that it may provides the potential for a pathway specific monitoring and intervention algorithm.

9. SUMMARY AND PERSPECTIVE

It has been well demonstrated by us and others that alterations of actin remodeling play a pivotal role in regulating the phenotypic and morphological events occurring during malignant transformation process. As shown in Figure 7, the critical level of regulation appears to be at the level of actin polymerization, in which an increased actin polymerization as indicated by an increased relative F/G-ratio in a giving cell is a marker for cellular differentiation. Conversely, a decreased F/G-actin ratio (increased G-actin or decreased F-actin) represents cellular dedifferentiation and malignant transformation. These observations provided a foundation for a hypothesis that specific molecular expression profiles of actin remodeling genes may be associated with specific malignant phenotypes such as tumor initiation, progression, and invasion. These expression profiles can then be utilized to define an individual's risk of bladder cancer in a much more specific and focus manner. For example, the initiation profile can be used to monitor the high-risk individual for developing cancer (early detection profile), whereas the progression and invasion/metastasize profile can be used as markers for prognostic indication (prognosis profile). Furthermore, targeted interventions can be carried out on specific actin signaling pathways in an effort to reduce the risk of either developing tumor or halt the progression of tumor development. The conceptual framework is depicted in Figure 8.

It is apparent that a hypothesis-driven approach based upon testing each gene singly would be inferior to functionally based, comprehensive analysis of genes involving in actin remodeling during transformation for providing a clear understanding of how actin is associated with the malignant phenotypic changes. Recent technological advances in molecular profiling analysis at different molecular levels (DNA, RNA, protein) enable this

goal to be reachable. This laboratory has recently carried out studies in this endeavor, and the preliminary results obtained thus far support our hypothesis.

10. ACKNOWLEDGEMENTS

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