# miRNAs, Genes and Cancer Cytology

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Histology and cytology are descriptive methods of ascertaining cancer cells. Genetic profiling is another means to accomplish this. The question is; how does the genetic change in a cancer cell get reflected in its morphology? We examine this with some insights from recent literature. 

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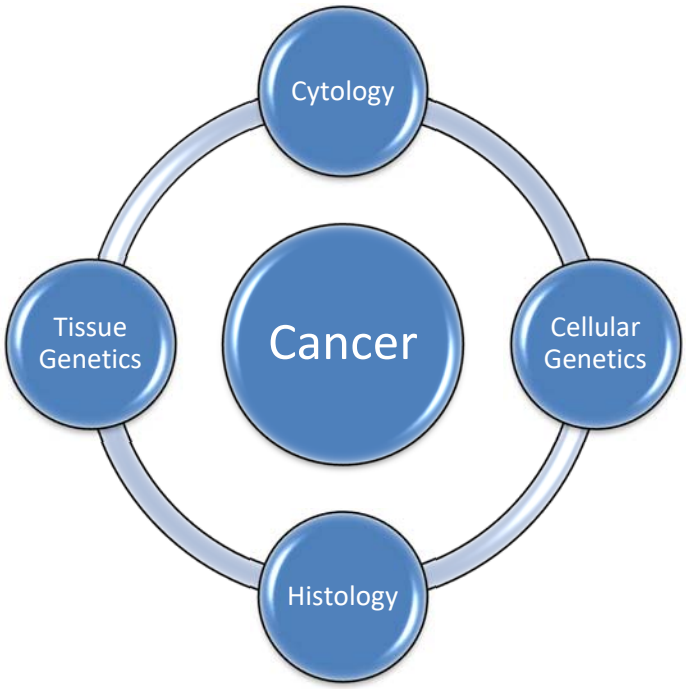
## Table of Contents

# 1 INTRODUCTION

Cytology concerns itself with a single cell and histology with an aggregate of cells. That is a well-known fact in medical studies. The genomics of a cell has been studied in depth with more being known most likely daily if not more often. The genetic structure of a collection of cells, however, seems to have been less examined. Thus the nexus between cytology and genomic cell architecture can be posited. We look at cancers on a cell by cell basis when it comes to genetic studies. We do not have a paradigm for the same in a histological context.

For example, in glandular cancers such as prostate, breast, thyroid we see the basal layers changing but when we examine the cytology, we see one set of things and when we look at the histology, we see another. We need, in my opinion a nexus paradigm for histological studies. The graphic below presents the necessary completion with some form of tissue genetics. Namely, the genetic description of the tissue as a system, not as a cell.



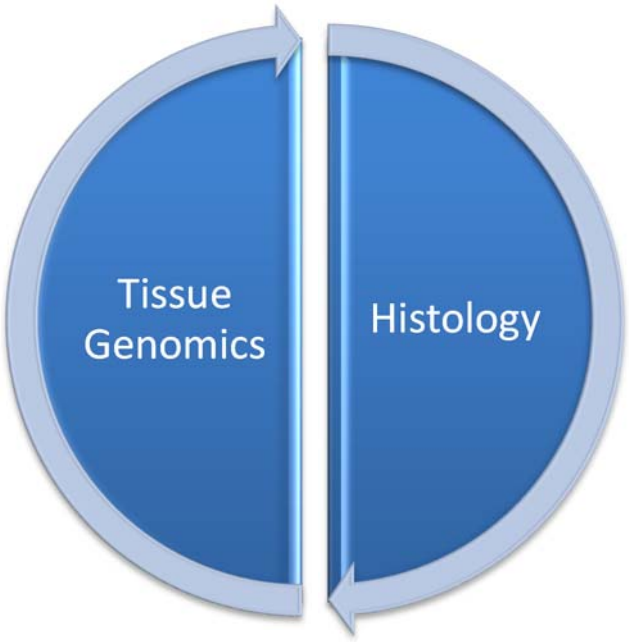

Now let us consider a recent example. In a paper by Boos et al, the authors have noted:

*Five percent of papillary thyroid carcinomas (PTC) show an adverse clinical outcome (ACO). The tall cell variant of papillary thyroid carcinomas (TCV) is a good predictor of an ACO, however, the identification of tall-cells is subjective. Micro RNAs are short non-coding ribonucleic acids (miRNA). Their expression in PTC could be a powerful, more objective predictor of prognosis. …One hundred and forty-nine miRNAs were significantly associated with an ACO, seventy-one of them with TC-morphology. Twenty-two miRNAs were identified as*

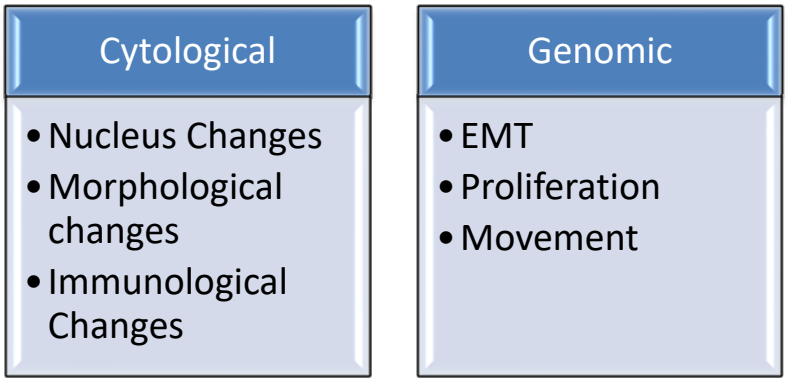
*targets for VEGF and thirty-two as targets for PTEN. In univariate and multivariable analysis, reduced expression of PTEN and an increased expression of VEGF were associated with shorter relapse free survival. A classifier, including TC-morphology, pT-stage, VEGF, and PTEN, predicted relapse with an 80% accuracy. … Some miRNAs predict outcome in PTC and are involved in TC-morphology in PTC. These miRNAs may serve as more objective indicators of an ACO than tall cell morphology. PTEN and VEGF protein expression are prognostically relevant and are at least partially regulated by miRNAs*

Here we have an interesting example of what we are talking of. We have a histological cancer, PTC, identified by single cellular artifacts, cytological, yet assembled in a manner to meet a histological criterion, and possessing a cytological phenotype, namely a tall cell. That is the cell is longer than wider. Now we have a concomitant genetic profile of the cancerous cells including key pathway elements but also including miRNAs. We are missing the Tissue Genomics, but we have the Cell Genomics. This begs the question: is the tall call variant the result of some cytological genomic variant, some histological genomic variant, or something else? What is the cause of the tall cell and can we relate it to some specific genomic system? This paper by highly respected pathologists presents both sides but lack a nexus. We will examine this a bit in the report.

Identifying cancers from histological analysis has been done for well over a century and it continues to evolve and get more refined. Generally, what is examined is the phenotypic characteristics of the cells and their individual presentations. Recently we now have the ability to examine the genotype of a cell and from that ascertain it malignant status. The question we examine is; will phenotype and genotypes merge or will the genotypic analysis succeed in the long term?



The challenge can initially be addressed with cytological observations versus genetic changes. Take the example of papillary thyroid cancer. It is identified by looking at the cell and seeing whether the nucleus is large, grooved, rough edged cells, tall cells and the like. The other side we know that papillary cancer cells have genetic disturbances such as RET, BRAF V600 and the like. The simple question which can be posed is; what are the genetic causes of the morphological changes in a cell to provide cytological changes and are they linked as cause and effect and if so how. Or, are we just seeing concomitant effects that arise in such a malignancy?



# 2 CELLULAR STRUCTURE

The question is: how do genes determine cellular structure. Or in some general sense; morphology recapitulates genology? The old adage of "ontogeny recapitulates phylogeny" has a certain merit but has been dismissed. Here we ask the question which relates shape and form to underlying mechanisms which we are aware of in cells. Perhaps we could replace morphology with cytology and even perhaps address the question of histology.

In cancers we see cells change shape, they change from epithelial to mesenchymal. We have examined that change in some detail elsewhere[1]. In other cancers they lose binding such as in melanoma[2]. The melanoma case is the case where we have a histological change with melanocytes moving from the normal basal layer. In prostate cancer we have proliferation and the initial assembly of morphologically similar but aberrant glands, excess cells and loss of structure[3]. Finally, in thyroid cancers we have nuclear changes that identify papillary cancers as well as histological changes in both papillary and follicular cancers[4]. These are but a few examples. In almost all cases we have no significant match between morphology issues and the underlying genetic deficits. Our intention is not to solve this issue, it is quite vast, but to articulate some of its dimensions and analyze the current literature. Again, this is but a first step in that direction and is no way definitive.

## 2.1 CELLULAR SHAPE

The shape of a cell and its spatial cohesiveness are key factors in assessing a malignant state. However, the shape of a cell and its cellular environment is dependent on the organ and the stage of its development. Developmental Biological factors determine what the cell will become and what its function is and how it relates to its environment. At the current state of understanding the detailed genetic progressions leading to say a kidney or liver cell and its environs is still a question of interest. Namely the natural progression of the embryo to specific organ and in turn their cells is a complex and yet to be fully understood area.

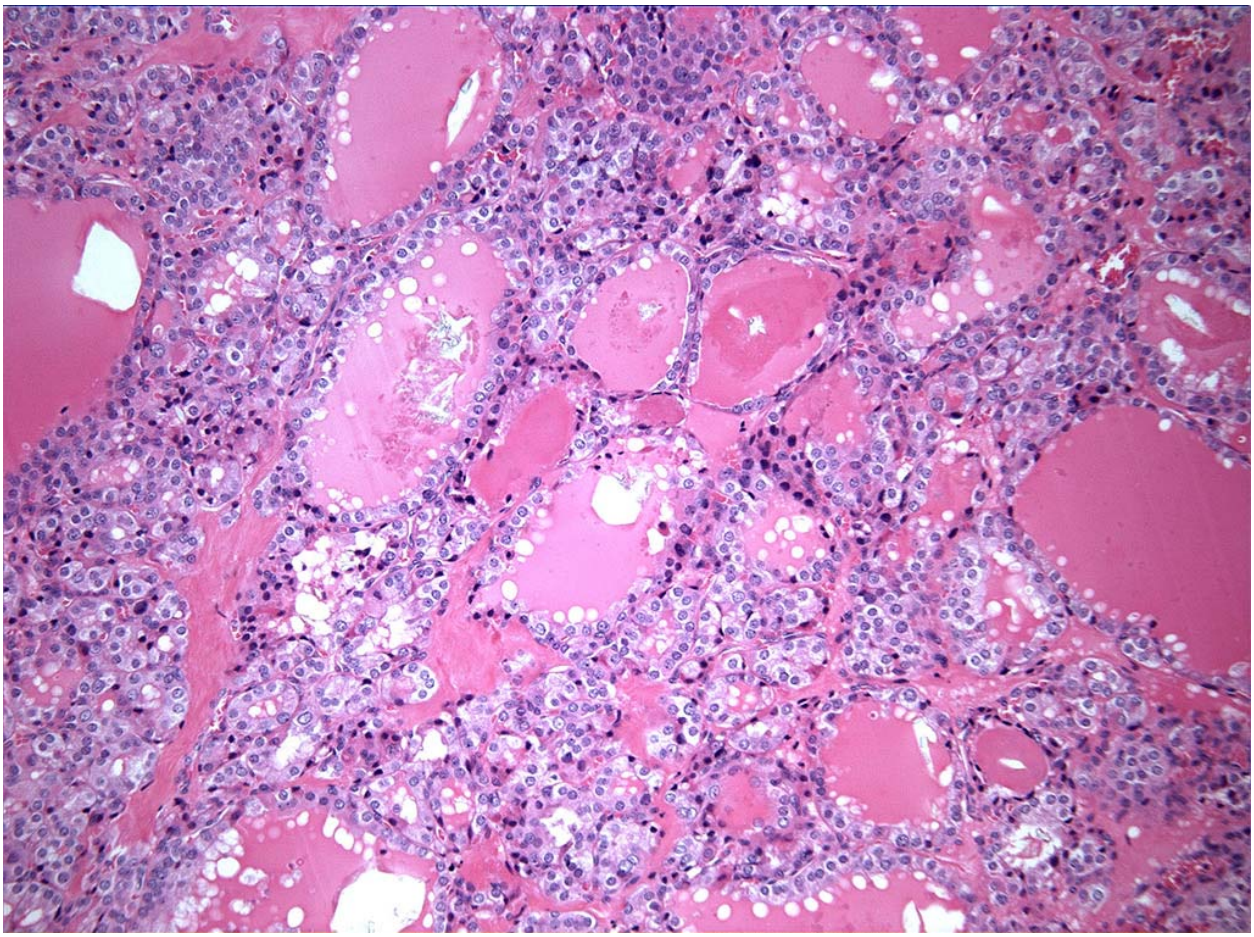
However, understanding the "normal" or "healthy" cell is a matter of observations and variants therefrom can then be examined for malignant behavior. For example, here is a normal thyroid.

[1] https://www.researchgate.net/publication/330222973_EMT_and_Cancers
and
https://www.researchgate.net/publication/333704252_EMT_lncRNA_TGF_SMAD_and_Cancers

[2] https://www.researchgate.net/publication/264960157_Melanoma_Genomics

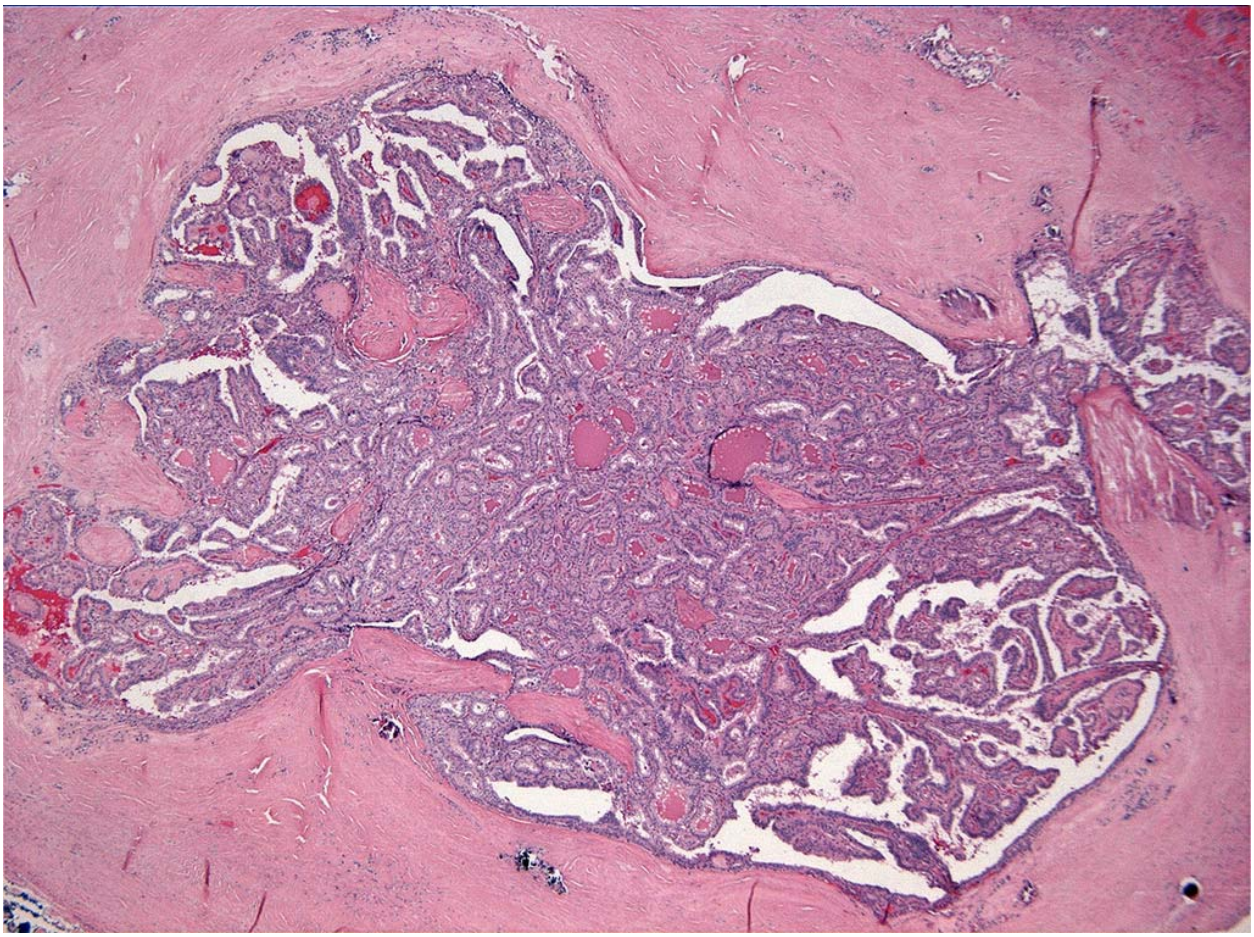
[3] https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach

[4] https://www.researchgate.net/publication/331935614_Thyroid_Cancer_Seek_and_Ye_Shall_Find

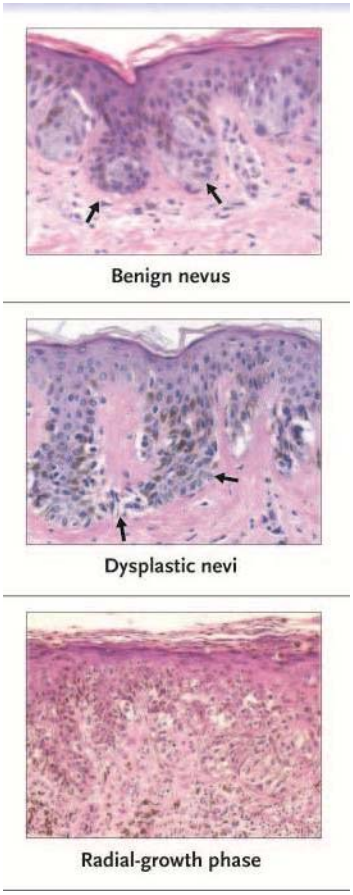


The glandular elements are shown with the internal colloidal sections from whence the T3 and T4 are produced and then sent out to the blood stream. (TSH enter the follicular cell which produces Thyroglobulin which enter the colloidal content and produces T3 and T4) The epithelial cells are follicular cells and the interstitial cells lie the in between areas along with vascular inclusions.

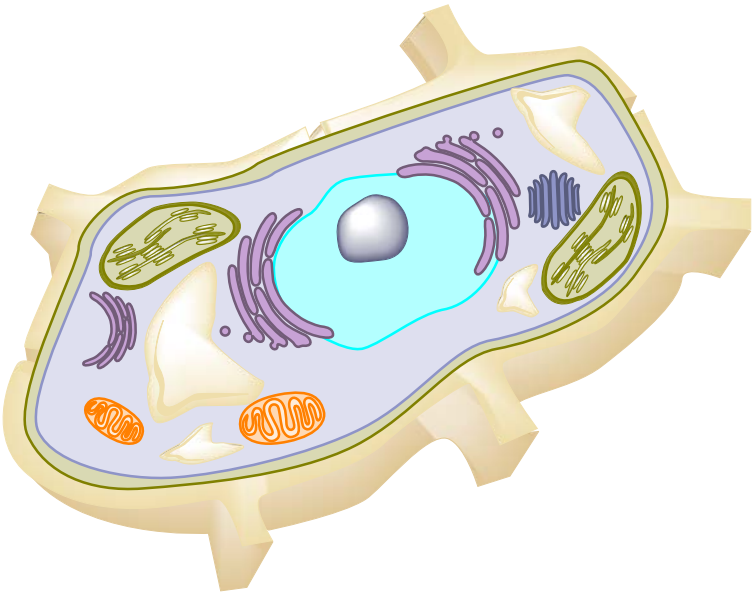
Now a papillary microcarcinoma is shown below:



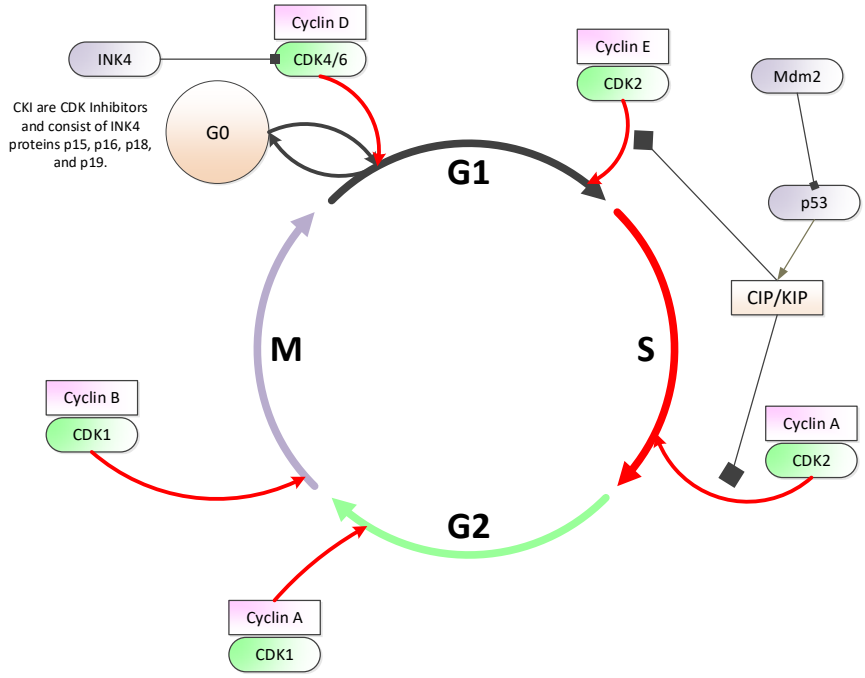
Note the loss of structure and the collection of cells in clumps. The arrangement of cells is one descriptive to identify a malignant growth. Consider a malignant melanoma. Below we demonstrate an example. The top is a benign nevus showing a proliferation of melanocytes but contained in the nasal layer. Then a dysplastic nevus with a significant proliferation and finally the superficial spreading melanoma in radial phase. In all cases we have identifiers as regards to the identification of the lesion. We do not look at the cell qua cell but the cell as collective.




The second major way to determine malignancy is to examine the cell qua cell. The prototypical cell and its key parts is depicted below. Namely we can examine a specific cell and from that examination determine if it contains aberrant form or structures. But the question still is: are those forms and structure dispositive and what are the underlying genetic changes which cause that morphological aberration? That is; is every cell with feature X a malignant cell? Namely, can that cell result in metastatic behavior?



The shape of the cell is also dependent on the state in the cell cycle. As shown below, we have the classic cell cycle:




The chromosomes are opening the replicating and thus the nucleus enlarges, expands and results in duplication. The cell cycle is something which occurs in mitosis and mitosis is a common element in malignant cell proliferation.

We can then examine the cellular structure in a complex, a collection of cells making up the target organ. As Misteli has noted:

*A central question in modern cell biology is how large, macroscopic cellular structures are formed and maintained. It is unknown what determines the different shapes and sizes of cellular organelles, why specific structures form in particular places, and how cellular architecture is affected by function and vice versa.*

*Two fundamentally different mechanisms exist to generate macromolecular structures: self-assembly and self-organization. Whereas self-assembly involves the physical association of molecules into an equilibrium structure, self-organization involves the physical interaction of molecules in a steady-state structure.*

*For example, virus and phage proteins self-assemble to true equilibrium and form stable, static structures. In contrast, most cellular structures (i.e., the cytoskeleton, nuclear subcompartments, or exocytic and endocytic compartments) are open for exchange of energy and matter and are governed by steady-state dynamics.*

*The concept of self-organization is based on observations of chemical reactions far from equilibrium, and it is well established in chemistry, physics, ecology, and sociobiology. Self-organization in the context of cell biology can be defined as the capacity of a macromolecular complex or organelle to determine its own structure based on the functional interactions of its components. In a self-organizing system, the interactions of its molecular parts determine its architectural and functional features. The processes that occur within a self-organized structure are not underpinned by a rigid architectural framework; rather, they determine its organization.*

As Mattick has noted:

***The developmental ontogeny of a human from an embryo to a fully formed adult involves the construction of an organism of approximately 100 trillion cells, with an extremely precise architecture and many differentiated tissues.*** *These include intricately sculpted bones, organs and muscles, such as the dozens of fine muscles in the face, as well as a brain that evolves in situ in response to experience. This is an extraordinary feat of genetic programming, which in all likelihood, requires enormous amounts of information.*

*This information directs not just a human developmental program, or that of another species, but the idiosyncrasies of the particular program that was inherited by the individual from their parents and their ancestors, as exemplified by the shape of our nose, mouth and ears and other identifying familial features. How is this feat achieved, and where is this information embedded? In the only well-studied case, the nematode worm Caenorhabditis elegans, it is known that developmental ontogeny is precise and invariant, with each cell in the adult being the result of a spatially and temporally ordered progression of cell division, selected apoptosis (programmed cell death) and, ultimately, differentiation into nerve, muscle, gut, germ and other specialized cells . Similar processes are observed in the development of insects and mammals, for example in the apoptosis that sculpts the eye ommatidia in the former and separates the digits of the fore- and hindlimbs in the latter.*

*Thus, it is likely that the ontogeny of higher animals, while vastly more complex and likely to be subject to individual (genomic) variation, is also precisely programmed. Indeed, the almost exact identity of monozygotic twins in their physical characteristics and idiosyncrasies, as well as a*

*high degree of concordance in their psychological characteristics (independent of environment), is clear testimony to the precision and reproducibility of the genetic instructions they share. The genetic programming of development is usually considered to be directed by proteins involved in morphogenetic signalling and various aspects of gene regulation.*

*These include homeodomain-containing proteins, chromatin-modifying proteins, and transcription factors acting on cis-regulatory elements, informed by those involved in cell surface receptor and signal transduction systems. Together they form elaborate modular regulatory networks – notwithstanding the recent discovery of microRNAs (see below) that are regarded as an interesting extension of the current paradigm rather than the vanguard of another entire layer of regulation. This protein-centric perspective underpins most conceptions of the control of development, as exemplified by elegant studies on sea urchin embryogenesis and fruitfly development*

The single cell generally has a characteristic shape and form. Epithelial cells in glands are surrounding the gland and the internal part of the gland is separate from the external. Blood flows to the gland and inter-glandular tissues provide the fabric for structural integrity. The problem is that there are so many types of normal cells that identifying an abnormal cell is based generally on a comparative analysis.

As Halbleib and Nelson have noted:

*Tissue morphogenesis during development is dependent on activities of the cadherin family of cell–cell adhesion proteins that includes classical cadherins, protocadherins, and atypical cadherins. The extracellular domain of cadherins contains characteristic repeats that regulate homophilic and heterophilic interactions during adhesion and cell sorting. Although cadherins may have originated to facilitate mechanical cell–cell adhesion, they have evolved to function in many other aspects of morphogenesis.*

*These additional roles rely on cadherin interactions with a wide range of binding partners that modify their expression and adhesion activity by local regulation of the actin cytoskeleton and diverse signaling pathways. Here we examine how different members of the cadherin family act in different developmental contexts, and discuss the mechanisms involved.*

A recent study by Aizarani et al examining liver cells noted:

*The human liver is an essential multifunctional organ. The incidence of liver diseases is rising and there are limited treatment options. However, the cellular composition of the liver remains poorly understood. Here we performed single-cell RNA sequencing of about 10,000 cells from normal liver tissue from nine human donors to construct a human liver cell atlas.*

*Our analysis identified previously unknown subtypes of endothelial cells, Kupffer cells, and hepatocytes, with transcriptome-wide zonation of some of these populations. We show that the EPCAM+ population is heterogeneous, comprising hepatocyte-biased and cholangiocyte*

*populations as well as a TROP2int progenitor population with strong potential to form bipotent liver organoids. As a proof-of-principle, we used our atlas to unravel the phenotypic changes that occur in hepatocellular carcinoma cells and in human hepatocytes and liver endothelial cells engrafted into a mouse liver. Our human liver cell atlas provides a powerful resource to enable the discovery of previously unknown cell types in normal and diseased livers…*

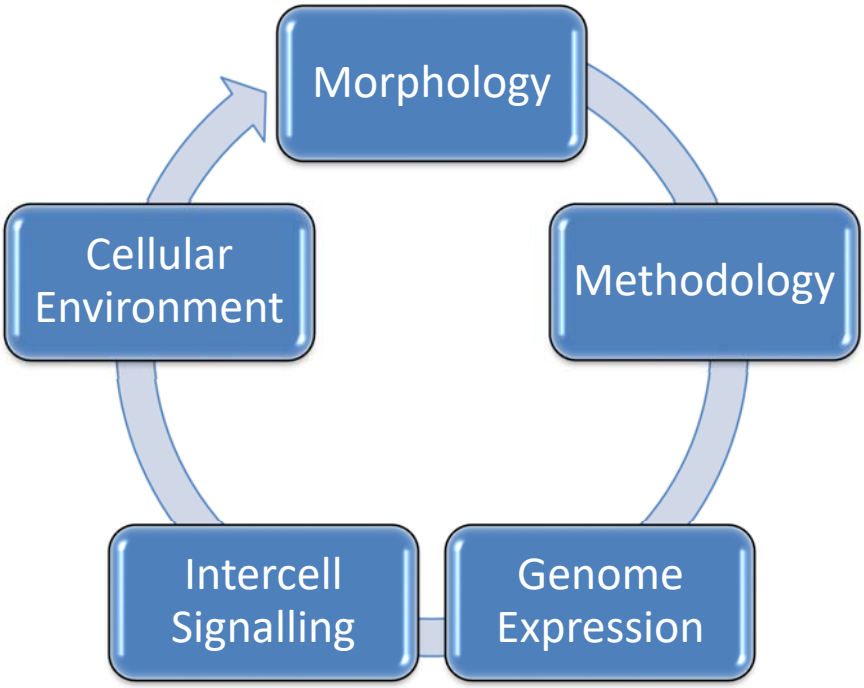
This is clearly an interesting and useful study. Namely by focusing on RNA on a cell by call basis, for 10,000 cells, one can see the significant disparity of the organ. How that disparity relates to morphological characteristics is yet to be determined. However this study provides boundary conditions on the genome expression side. The authors continue:

*Hepatocytes are spatially heterogeneous and zonated along the portal–central axis of the liver lobule. According to metabolic sub-specialization, the liver lobule has been divided into the periportal zone surrounding the portal triad (portal vein, hepatic artery and bile duct), the central zone nearest to the central vein, and the remaining mid zone. Whereas previous observations have suggested that non-parenchymal cells such as LSECs and Kupffer cells have specialized subtypes, it has been hard to demonstrate heterogeneity of these cell types, and most studies have been carried out in rodents…*

Again getting down to the specific cell level is useful. However, the mechanism operating between DNA, RNA, and in turn the cell morphology is missing. They conclude:

*We have established a human liver cell atlas, revealing heterogeneity within major liver cell populations and the existence of an epithelial progenitor in the adult human liver. Our atlas reveals transcriptome-wide zonation of hepatocytes and endothelial cells, and suggests that different liver cell types may cooperate to carry out essential functions. Although we could validate predicted zonation profiles with antibody staining, it will be essential to perform more large scale in situ gene expression analysis.*

In summary, there is no well-established method the correlate morphology, mechanism and gene expression. In addition, it may be most likely an environmental factor relating to cell location as well as intercell signalling. We graphically depict this below:




## 2.2 NUCLEAR SHAPE

Nuclear shape is also a marker for many cancers. The nucleus is a significant player in many cancers if not all. One can look at the nucleus as two major elements; chromosomes and in turn the genes, and second, the nucleolus.

As Gartner and Hiatt describe the nucleus:

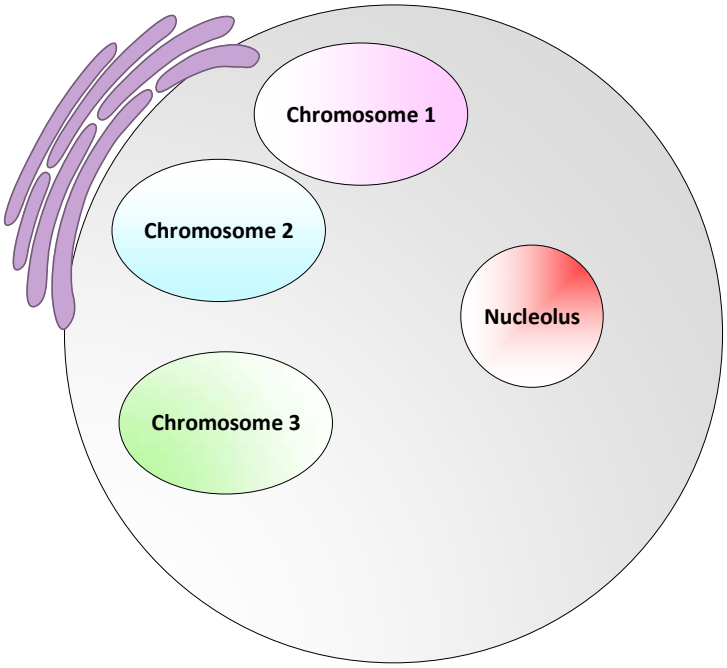
*The nucleus is the largest organelle of the cell. It contains nearly all of the deoxyribonucleic acid (DNA) possessed by the cell as well as the mechanisms for ribonucleic acid (RNA) synthesis, and its resident nucleolus is the location for the assembly of ribosomal subunits. The nucleus, bounded by two lipid membranes, houses three major components: Chromatin, the genetic material of the cell and the nucleolus, the center for ribosomal RNA (rRNA) synthesis.*

*Nucleoplasm, containing macromolecules and nuclear particles involved in the maintenance of the cell. The nucleus is usually spherical and is centrally located in the cell; however, in some cells it may be spindle-shaped to oblong-shaped, twisted, lobulated, or even disk-shaped. Although usually each cell has a single nucleus, some cells (such as osteoclasts) possess several nuclei, whereas mature red blood cells have extruded nuclei.*

*The size, shape, and form of the nucleus are generally constant for a particular cell type, a fact useful in clinical diagnoses of the degree of malignancy of certain cancerous cells.*

*The nucleus is surrounded by the nuclear envelope, composed of two parallel unit membranes: the inner and outer nuclear membranes, separated from each other by a 10- to 30-nm space called the perinuclear cisterna. The nuclear envelope is perforated at various intervals by nuclear pores that permit communication between the cytoplasm and the nucleus. At these pores, the inner and outer nuclear membranes are continuous with one another. The nuclear envelope helps to control movement of macromolecules between the nucleus and the cytoplasm and assists in organizing the chromatin.*

The diagram below gives a simplistic graphic for the nucleus. Generally it is an enclosed element with wrapped chromosomes, separated from one another and containing a nucleolus.




The nucleus often shows various aberrations in malignant cells (see Pajerowski et al). As Webster et al note:

*The nuclei of most cells are either round or oval. This, in itself, is hardly remarkable except for the fact that various diseases, as well as aging, are associated with alterations in nuclear shape. Moreover, in certain specialized cell types, altered nuclear shape is important for cell function. But what determines nuclear shape, and how does shape affect function? In many cell types, altered nuclear shape is due to changes in the nuclear lamina. In some cases, however, the shape of the nucleus is altered by forces that act from the cytoplasm. In either case, it is still not entirely clear how nuclear shape affects function, although two main hypotheses exist.*

*The first hypothesis posits that changes in nuclear shape alter the rigidity of the nucleus; this could be beneficial for cells that need to squeeze through tight spaces, but deleterious to cells that are under mechanical duress. The second hypothesis proposes that changes in nuclear shape result in chromatin reorganization and thereby affect gene expression. It is important to*

*note that these two hypotheses are not mutually exclusive. In addition, because nuclear shape changes are often accompanied by an altered nuclear lamina, it is possible that the dramatic effect on cell function is due to aberrant properties of the lamina rather than nuclear shape changes per se. In this section, we examine some of the cell types and conditions that are associated with irregular nuclear shape, and we discuss, when known, the causes of these shape changes and how they affect cell function…*

*There are conflicting reports regarding the dominant cellular factors that determine nuclear volume. One idea, known as the nucleoskeletal theory, is that DNA content influences the volume of the nucleus, which in turn influences the size of the cell. Intuitively, DNA may affect nuclear volume, because the size of the nucleus could be directly proportional to amount of DNA it contains and the extent to which that DNA is compacted. Simply comparing genome size to nuclear and cell volume among species supports this theory, because species with larger genomes generally have larger nuclear and cellular volumes. Experiments in mice also give credence to the nucleoskeletal theory: it has been shown that tetraploid mouse embryos have nuclei that are twice as large as those in a diploid control. However, other data suggest that genome size per se is not the determining factor of nuclear size.*

*Rather, it is likely that there is a nuclear-scaling mechanism whereby nuclear volume is proportional to, and determined by, the levels of one or more cellular factors. Indeed, nuclear transplant experiments support this claim: implanting a small hen erythrocyte nucleus into a HeLa cell results in expansion of the nucleus to the appropriate size for its new environment, without affecting DNA content. Moreover, the nucleoskeletal theory does not explain why cells from different tissues in a given organism have the same amount of DNA but varied nuclear sizes. Studies in yeast also contradict the notion that DNA content dictates nuclear and cellular volumes. In neither fission yeast nor budding yeast does nuclear volume increase sharply during S phase, as would be expected if DNA content had a direct affect on nuclear size. Furthermore, even a 16-fold increase in ploidy does not affect nuclear size in fission yeast.*

*Instead, the displacement of nuclei by centrifugation in multi-nucleated fission yeast showed that nuclear size adjusted in proportion to the amount of surrounding cytoplasm. These studies support a mechanism whereby nuclear size is determined by cytoplasmic volume rather than DNA content. Assuming that cytoplasmic factors determine nuclear size, what might these be? In cell-free extracts of Xenopus oocytes, an increase in nuclear volume after NE reassembly requires an intact ER. This suggests that the membrane for the newly formed NE is supplied by the ER, and therefore membrane availability could be a limiting factor in determining nuclear size. The ER exists as a continuous meshwork of membrane sheets and membrane tubules.*

*Proteins known as reticulons cause tubule formation in the ER (Voeltz et al., 2006), and high levels of reticulons are inhibitory to nuclear growth, which suggests that the availability of membrane in the form of sheets can put an upper limit on nuclear size (Anderson and Hetzer, 2008; Kiseleva et al., 2007). Work in the Xenopus system has demonstrated a requirement for NPCs and nuclear import in nuclear growth after NE assembly (D'Angelo et al., 2006; Newport et al., 1990), which suggests that the import of one or more nuclear proteins contributes to sizing the nucleus. Indeed, several nuclear lamina proteins that are transported into the nucleus through the NPCs have been found to affect interphase nuclear growth (e.g. Brandt et al., 2006; Dittmer et al., 2007; Newport et al., 1990). However, many questions remain. For example, how*

*do yeast – which lack lamins and lamin-associated proteins – adjust nuclear volume in response to changes in cytoplasmic volume? Also, what is the mechanism, in any organism, that establishes the upper limit to nuclear growth? Does size matter for nuclear function? Although the mechanisms that control nuclear volume remain unclear, the existence of a karyoplasmic ratio suggests that nuclear size is important for cell function. Disturbance of this ratio is associated with certain types of cancers (Slater et al., 2005; Zink et al., 2004), suggesting that the ratio between nuclear and cytoplasmic volumes is crucial for cell integrity. Moreover, it has been proposed that cell-cycle progression depends on nuclear size (Roca-Cusachs et al., 2008; Yen and Pardee, 1979), and that cells monitor the ratio between cytoplasmic and nuclear volume to gauge… The existence of diseases associated with altered nuclear shape and size underscores the importance of uncovering the mechanisms that control NE dynamics. In the past few years, the field of nuclear architecture has witnessed an explosion of knowledge, from the understanding of how nuclear lamina proteins function to the basic principles of NE assembly. We still need to determine the link between nuclear shape and nuclear function, and to distinguish between cases where an altered cellular function is a direct consequence of altered nuclear shape and those where both altered function and shape are independent consequences of defects in a particular structure (e.g. the nuclear lamina). Equally interesting is how nuclear size is determined; although a link between nuclear size and cytoplasmic volume has been suspected for many years, recent studies in yeast have introduced a tractable genetic system in which this question could be answered.*

*Likewise, mutant and RNAi screens in other organisms could uncover proteins that are involved in nuclear size determination. Finally, the finding that NE assembly begins with ER tubules, rather than vesicles, has opened a new avenue of investigation that is focused on understanding the properties of the ER and of the proteins that contribute to ERmembrane dynamics.*

*In the coming years, it is likely that we will learn more about the relationship between the ER and NE dynamics; specifically, how the balance between membrane tubules and membrane sheets is maintained and regulated, and how lipid synthesis contributes to both ER and NE structure.*

## 2.3 NUCLEOLUS

The nucleolus is the "factory" in the nucleus for ribosome production and a variety of other functions. The nucleolus has long been recognized as a significant player in identifying malignant cells.

Lam et al describe the nucleolus as:

*The nucleolus is the most prominent structure in a cell nucleus. It is the site of ribosomal RNA (rRNA) transcription, pre-rRNA processing and ribosome subunit assembly. The nucleolus is a dynamic structure that assembles around the clusters of rRNA gene repeats during late telophase, persists throughout interphase and then disassembles as cells enter mitosis. Owing to*

*the difference in density between the nucleolus and the surrounding nucleoplasm, it is readily visible in either live or fixed cells viewed by phase contrast or differential interference contrast (DIC) optics.*

*Thanks to the advent of fluorescent protein (FP) technology, nucleoli can also be detected by fluorescence microscopy in cell lines expressing FP-tagged nucleolar proteins. An example is shown in the inset of the upper-left panel, in which PP1γ, a protein phosphatase that accumulates in the nucleolus, is tagged with YFP and stably expressed in HeLa cells.*

*Like all other intranuclear structures, the nucleolus is not membrane enclosed, but the combination of its unique density and robust structure makes it one of the most convenient subcellular structures to purify.*

*Thus, when mammalian nuclei are physically disrupted (e.g. by sonication) in a solution of low salt concentration, nucleoli remain intact even under conditions that disintegrate most other subnuclear structures.*

*Nucleoli can therefore be isolated in essentially pure form by centrifuging sonicated nuclei through a density cushion. The isolated nucleoli are intact, similar in size and morphology to the nucleoli in live cells and even retain transcriptional activity to some extent*

Identifying malignancies via examining the nucleolus has been common for the past century or more. Penzo et al have noted:

*Pathologists had focused on the nucleolus as a parameter for the diagnosis of tumour malignancy since the end of the nineteenth century, and hypertrophied nucleoli were considered a hallmark of cancer cells. However, whether the size of the nucleolus actually constituted a reliable parameter for distinguishing malignant from benign tumour tells remained undefined for a long time.*

*In 1986, Ploton and colleagues succeeded in visualizing the interphase AgNORs by light microscopy in routine paraffin sections by applying t modified, simple silver staining method for the NOR proteins. Using; this method, the AgNORs appear at well-defined black dots distributed within the nucleolar body and perfectly identify the structural-junctional units; of the nucleolus…*

*This series of studies concluded that malignant tumors generally have larger nucleoli than corresponding benign lesions of the same tissue. However, in many malignant tumors, such as breast cancer, thyroid cancer, cervical intraepithelial neoplasia, stomach cancer and endometrial cancer cells have nucleoli whose size overlaps that of the corresponding normal - issues and benign tumor lesions of the same histotype.*

*The absence of any nucleolar since overlap was demonstrated only between naevocellular naeve and melano-carcinomas and in pleural effusions between mesothelioma or metastatic cells and reactive cells. 'Therefore, with these excrdions, nucleolar size con help to distinguish a*

*malignant from a benign tumor but cannot be generally considered to represent an absolute diagnostic parameter in tumor pathology…*

*Alterations in nucleolar size, is recognized as valuable prognostic marker in many different kinds of tumours. In addition, the visualization of nucleolar patterns in routine histological analysis may give important hints on the prognosis of the malignancies, and possibly also valuable information on the advisability of ribosome-biogenesis targeting therapies. In particular, for those p53-competent tumours with nucleolar hypertrophy (and, therefore, with up-regulated ribosome biogenesis) a therapeutic approach selectively inhibiting rDNA transcription should be considered, evaluated on the basis of its clinical effect and possibly juxtaposed to other chemotherapies.*

The nucleolus has several distinct regions. As Pederson notes:

*Its three classical regions are defined by the different appearance of intranucleolar regions when viewed by electron microscopy (Fig. 3). These are the fibrillar centers, the surrounding dense fibrillar component, and the granular component. Few in the field anticipated the controversies that would ensue when several labs tried, logically enough, to link the sites of rRNA transcription and processing and assembly into nascent ribosomes with these three EM-defined nucleolar zones…Recent work has indicated that one of the three nucleolar regions, the granular component, is itself composed of at least two distinct molecular domains. ..*

*Beyond the possible roles of the nucleolus in cell-cycle control, viral replication, stem cell biology, and cellular senescence, there is another potential nucleolus function that warrants consideration, both for its historical precedence and its now reasonable plausibility based on a number of studies. This is the possibility that the nucleolus is involved, somehow, in the production of messenger RNA…The nucleolus is unique in that the discovery of one of its functions took far longer, namely more than a century, than was the case for most other cell components. Another half-century has brought the realization that the nucleolus is also the site of signal recognition particle biosynthesis, serves as a regulatory zone of cell-cycle progression mediators, and is a locus of mRNA and microRNA traffic, the functional meaning of which remains to be discovered. The nucleolus is now known to be a more dynamic domain of nuclear organization than once thought.*

Lamond and Earnshaw note:

*The nucleolus is formed around the ribosomal DNA (rDNA) repeats, which cluster at chromosomal loci called nucleolar organizers, and is the factory in which 28S, 18S, and 5.8S ribosomal RNAs (rRNAs) are transcribed, processed, and assembled into ribosome subunits. Nucleolus formation is both transcription- and cell cycle– dependent: In most eukaryotic cells, the entire structure breaks down and reforms during each mitotic cycle.*

*Thus, the nucleolus is a dynamic structure that forms in response to the requirement for new ribosome synthesis. Within the nucleolar factory, the rRNA is extensively modified during ribosome biogenesis…Small nucleolar ribonucleoproteins (snoRNPs) have been shown to act as "guide RNAs" during rRNA maturation, …directing nucleolytic cleavage of the rRNA precursor…Small nucleolar RNAs can be engineered to target 29-O-methylation to regions of*

*RNA that are normally unmodified. This approach should have important applications for studying how RNA modification influences RNA function.*

Montanaro et al note:

*The complex aspects linking the nucleolus and ribosome biogenesis to cancer are reviewed here. The available evidence indicates that the morphological and functional changes in the nucleolus, widely observed in cancer tissues, are a consequence of both the increased demand for ribosome biogenesis, which characterizes proliferating cells, and the changes in the mechanisms controlling cell proliferation.*

*In fact, the loss or functional changes in the two major tumor suppressor proteins pRB and p53 cause an up-regulation of ribosome biogenesis in cancer tissues. In this context, the association in human carcinomas of nucleolar hypertrophy with bad prognoses is worthy of note. Further, an increasing amount of data coming from studies on both hepatitis virus-induced chronic liver diseases and a subset of rare inherited disorders, including X-linked dyskeratosis congenita, suggests an active role of the nucleolus in tumorigenesis.*

*Both an up-regulation of ribosome production and changes in the ribosome structure might causally contribute to neoplastic transformation, by affecting the balance of protein translation, thus altering the synthesis of proteins that play an important role in the genesis of cancer.*

The authors continue regarding genetic drivers:

*The nucleolar changes of practically all human cancers have been evaluated and shown to be highly variable and independent of the histogenesis of the tumors as well as within the same tumor sample.26 Interestingly, even though nucleolar hypertrophy and functional upregulation of the nucleolus are generally considered to be a characteristic of cancer cells, these studies demonstrate that this was not always true: the nucleolar size and functional activity is sometimes lower than those of the corresponding normal cells.*

*In fact, the nucleolar changes in tumors are closely related to the number of proliferating cells within the cancer tissue and the rapidity of proliferation of the cycling cells, kinetics parameters that are highly variable in human tumors and sometimes of lower value than in the corresponding proliferating normal tissues.*

*There is now evidence that the upregulation of the nucleolar function occurring in proliferating cells is attributable to the products of the same proto-oncogene and tumor suppressor genes that control cell proliferation. Changes of proto-oncogenes and tumor suppressor genes occur very frequently in a variety of human cancers that are responsible not only for the loss of the normal control mechanisms of cell proliferation and cell cycle progression, but also for an enhanced ribosome biogenesis.*

*c-Myc, which is necessary and sufficient for cell-cycle entry, may be overexpressed in a variety of human hematological malignancies and solid tumors29 and directly enhances RNA*

*polymerase I transcription activity by binding to specific consensus elements of rDNA and recruiting the selectivity factor 1 (SL1) to the rDNA promoter.*

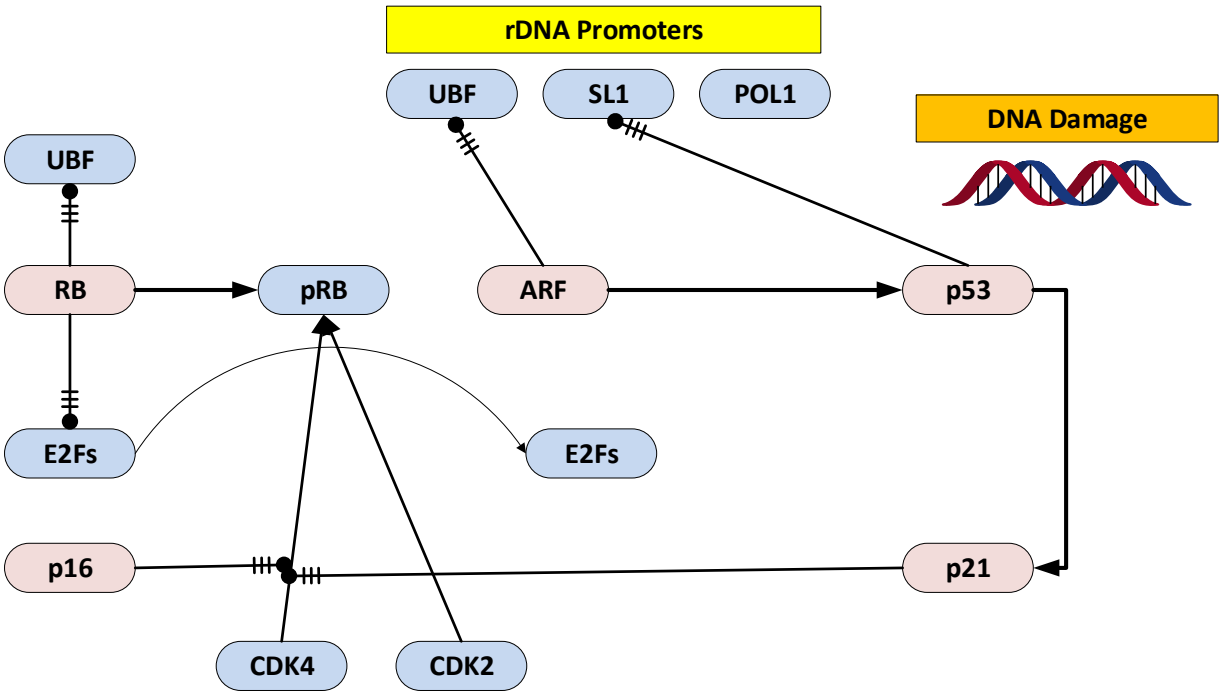
*In fact, SL1 is necessary for rDNA transcription by recruiting RNA polymerase I, in a complex together with the UBF, to the rRNA gene promoter.32 Furthermore, the Myc oncoprotein directly controls the transcription of several nucleolar proteins necessary for ribosome biogenesis.*

*Cyclin D and E, which control the normal cell cycle progression and which may be overexpressed or altered in a number of human tumors,34 also induce the phosphorylation of UBF by cyclin-dependent kinase 4 (Cdk4)-cyclin D1- and Cdk2-cyclin E mechanism, thus enhancing the transcription of ribosome genes.*

*However, among the genetic changes leading to neoplastic transformation that are of special importance for both their highly frequent occurrence and their effects on ribosome biogenesis, those involving the retinoblastoma tumor suppressor protein (pRB) and p53 pathways are undoubtedly the most relevant.*

The authors describe the key pathway elements as shown below and from their paper as modified:

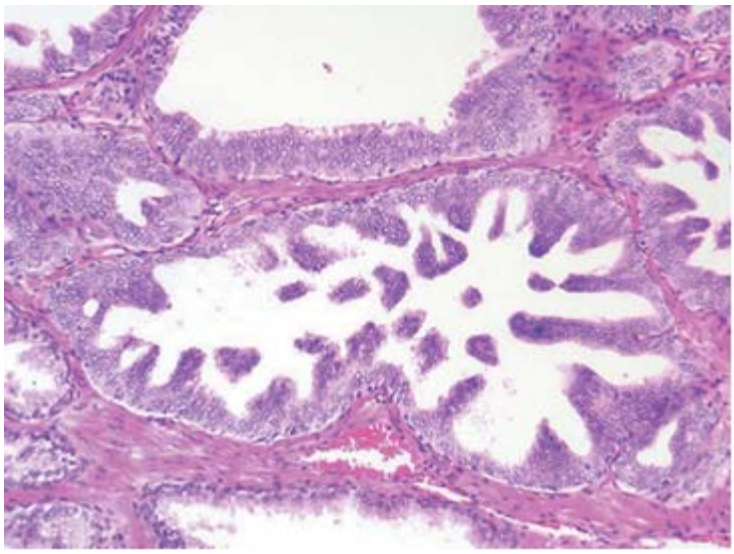
Montanaro et al




Simplified diagram of the control mechanisms of cell proliferation and ribosome biogenesis by pRB and p53. Inactive pRB is linked to UBF and E2Fs. When the cell nters the cell cycle, phosphorylation of pRB by cyclindependent kinases 4 and 2 occurs, freeing UBF and the E2Fs. UBF, together with SL1, binds to the rDNA promoter, thus allowing rDNA transcription by RNA polymerase I (Pol I). The E2Fs bind to their target genes, thus allowing DNA duplication and cell cycle progression. DNA damage stabilizes p53, which blocks ribosome biogenesis both by directly binding to SL1 and by hindering pRB phosphorylation through the inhibitory action of p21 on Cdk4 and 2. Cdk4 may be also constrained by p16Ink4a. Ribosome biogenesis may also be inhibited by Arf, which binds to SL1 and stabilizes p53.

## 2.4 HISTOLOGICAL SHAPE

What causes the change in the histological presentation of various cancers? Consider the HGPIN in the prostate as shown below:



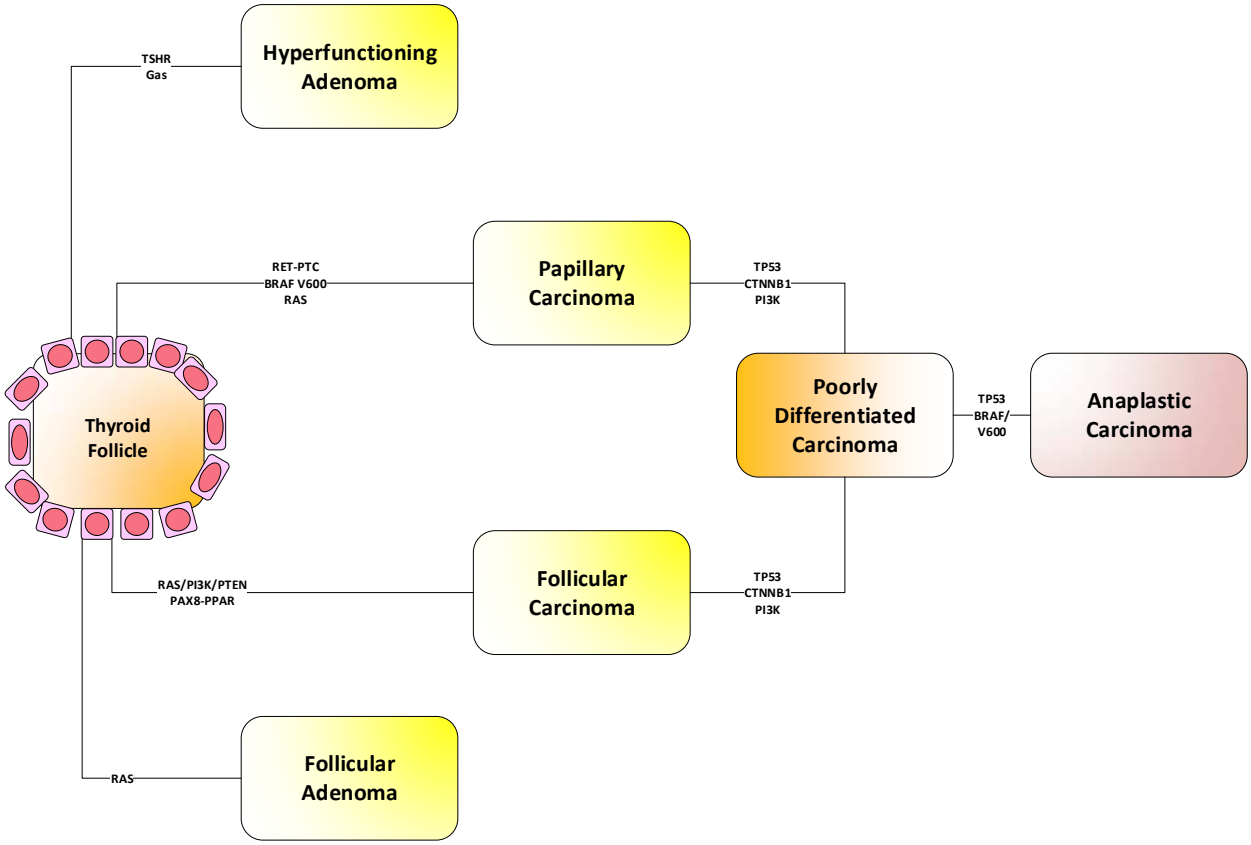


There is a proliferation of columnar cells on top of the basal cells in the glands. Instead of a simple glandular structure we have the beginnings of growth. One simple answer to this is that cell proliferation is occurring. Then we can examine the cell proliferation pathways and we can see there are several and that there may be a multiplicity of gene aberrations. This is often called a carcinoma in situ. Carcinoma because of the latent potential to metastasize. In situ because it is not expanding beyond its normal reach. The problem we have with this however is that there are times we see the HGPIN disappear. The question is; what caused the disappearance? There can be many possible answers, none of which may be correct at this time[5].

[5] https://www.researchgate.net/publication/325047485_PROSTATIC_INTRAEPITHELIAL_NEOPLASIA_PROGRESSION_REGRESSION_A_MODEL_FOR_PROSTATE_CANCER

# 3 THYROID CANCER

Examining thyroid cells for a malignant form can be challenging. For large tumors the histological analysis can be somewhat straight forward but for small lesions the identification of papillary carcinomas is based often upon nuclear issues. We demonstrate this progression in general terms below. In each we also link the genes that putatively impact the changes. Thus, RET is a major player in papillary transformation as is RAS and BRAF V600. In contrast PTEN plays a role in follicular. The former is a shape change and the latter a proliferation in terms of cell morphology. Finally, p53 is a key step moving to the more aggressive types of thyroid cancers.




## 3.1 OVERVIEW

In a somewhat classic paper by Suen and Quenville:

*In about 50% of our cases, the tumour cells exhibit intranuclear inclusions of the cytoplasm, so-called pseudonucleoli. Although commonly associated with thyroid papillary carcinoma, these cytoplasmic invaginations have been observed in other lesions including adenocarcinomas and melanoma. Twenty per cent of our aspirates contain psammoma bodies. These are basophilic, laminated spherules measuring 30-100,um in diameter, and must not be confused with dystrophic calcifications which can be seen in involutional goitres and occur as irregular, basophilic speckles lacking distinct laminations. In 10% of our cases, a great number of*

*lymphocytes are admixed with the tumour cells. An erroneous diagnosis of chronic lymphocytic thyroiditis may be made by the unwary because the numerous lymphocytes can obscure the presence of tumour cells.*

This observation of almost 40 years ago presents a multiplicity of morphological characteristics of thyroid cancers. However, the recognition of lymphocytes were seen as a confusion to the pathologist rather than a possible diagnostic measure.

As Siironen notes in a Thesis:

*Microcarcinoma. Microcarcinoma is a papillary carcinoma 1 cm or less in diameter. These lesions are of very low malignancy, and distant metastases are exceptionally rare. The indolent behaviour of these lesions is shown by their frequency as incidental findings in autopsy studies, being 6 to 7% in USA, Portugal, and Sweden, and 35% in a study from Finland).*

*Follicular variant. The follicular variant of PTC is the most common subtype of PTC after the usual type. It is composed of follicles and has characteristic papillary type nuclei. Thus, diagnosis of PTC is made when papillae are absent. These lesions behave biologically similarly to the usual type. In addition to common follicular variant, two other types of follicular variant have been described. The macrofollicular variant is less aggressive and the diffuse follicular variant more aggressive than common follicular variant. The diffuse follicular variant occurs mainly in young females. In series of eight patients, all developed metastases in lungs, bones or both, two of them died of PTC. Diffuse sclerosing variant. This variant was initially described by Crile and Fisher in 1953. It occurs predominantly in young individuals and is characterized by diffuse involvement of one or both lobes, dense sclerosis, abundant psammoma bodies, typical papillary carcinoma elements, foci of squamous metaplastic change, and a patchy lymphocytic infiltrate. Metastases both to cervical nodes and lungs are more frequent than in the usual type.*

*Oncocytic variant (Hürthle cell PTC). Oncocytic variant is characterized by the presence of oncocytes (also called oxyphilic cells), which are large polygonal cells with hyperchromatic nuclei and an eosinophilic granular cytoplasm. This oncocytic variant may have a papillary or follicular architecture. Diagnosis is based on the classical nuclear features of papillary thyroid carcinoma. It seems to behave in a fashion analogous to the usual type of papillary carcinoma.*

*Tall cell variant. Tall cell variant is defined as PTC in which a minimum of 30% of the cells have a height at least twice their width, indicating a more aggressive growth pattern. The tall cell variant is associated with a higher incidence of recurrence and mortality. It occurs more often in older patients.*

*Columnar cell variant. Thyroid tumours with columnar features were first described by Evans in 1986. They are rare, aggressive and have high mortality The papillae are lined by tall columnar cells showing pseudostratifi cation. The nuclei are elongated or oval, and are rich in chromatin, unlike the usual type. Columnar cell variant is an aggressive tumour associated with a fatal outcome.*

Another variant is the NIFTP. Shrestha et al have characterized it as:

*The re-naming of noninvasive follicular variant papillary thyroid cancer to the apparently non-malignant, noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) impacts the prevalence of malignancy rates, thereby affecting mutation frequency in papillary thyroid cancer. Preoperative assessment of such nodules could affect management in the future. The original publications following the designation of the new nomenclature have been extensively reviewed.*

*With the adoption of NIFTP terminology, a reduction in the follicular variant of papillary thyroid cancer (FVPTC) prevalence is anticipated, as is a modest reduction of papillary thyroid cancer (PTC) prevalence that would be distributed mainly across indeterminate thyroid nodules.*

*Identifying NIFTP preoperatively remains challenging. RAS mutations are predominant but the presence of BRAF V600E mutation has been observed and could indicate inclusion of the classical PTC. The histological diagnosis of NIFTP to designate low-risk encapsulated follicular variant papillary thyroid cancers (EFVPTCs) would impact malignancy rates, thereby altering the mutation prevalence. The histopathologic criteria have recently been refined with an exclusion of well-formed papillae. The preoperative identification of NIFTP using cytomorphology and gene testing remains challenging.*

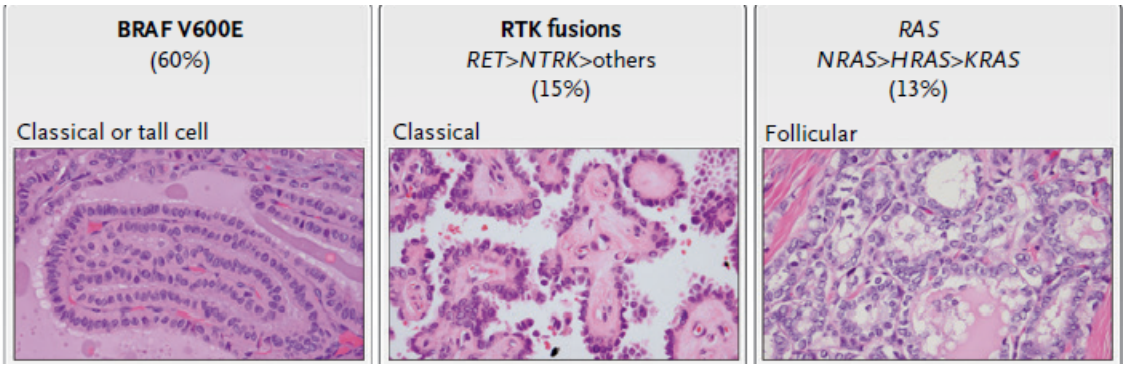
They go on to characterize it specifically as:

1. *Encapsulation or clear demarcation*
2. *Nuclear score 2–3*
3. *No vascular or capsular invasion*
4. *No tumor necrosis*
5. *No high mitotic activity (<3/HPF) Follicular growth pattern with:*
6. *<1% Papillae (criteria modified in 2018 to "no well-formed papillae") No psammoma bodies <30% solid/trabecular/insular growth pattern*

Thus, if one sees a micro FVPTC, fully encapsulated, no vascularization, and a singular lesion with no nodes, and no expression of fusion or genetic mutations, is this a carcinoma? Is morphology of the cells the telling sign, is the genetic profile more compelling, or what? We now examine some histological factors.

## 3.2 HISTOLOGY

We now will examine some of the key histological/cytological markers for papillary thyroid cancers. The following are examples of some gene-histology papillary thyroid cancers. The Tall Cell version is on the left and the classic papillary formation in the center and the follicular variant papillary thyroid cancer (FVPTC) on the right.



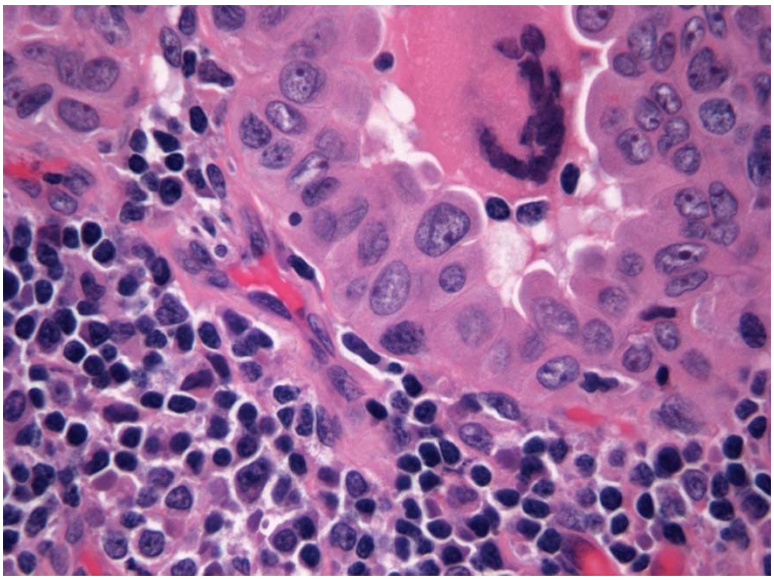

The question the above pose is; how are the genetic alterations related to the morphological changes? We now consider some specific details. The question still posed is: what are the genetic changes creating these effects? We now use the examples in the text by Boerner and Asa to demonstrate a few examples of the cellular artifacts used to identify papillary cancers. There are a multiplicity of factors and we use the most significant as a demonstrative.

### *3.2.1 Nuclear Enlargement*

As we noted previously, the nucleus general is a small fraction of most cells. The chromosomes are wrapped tightly and the nucleolus is relatively dormant. As the cell becomes more active one would expect the expansion of the chromosomes and thus an enlargement of the nucleus. Boerner and Asa note:

*The nuclei in thyroid neoplasia enlarge and do so out of proportion to any increase in cytoplasm, resulting in an increased nuclear/cytoplasmic (N/C) ratio. This nuclear enlargement may also be recognized by any of the following three manifestations of increased N/C ratio: Loss of the apparent basal polarization of the nuclei (seen histologically) Nuclear crowding and overlapping in the epithelial fragments (seen cytologically and histologically) Transition from monolayered sheets to syncytial aggregates (seen in cytological preparations)*

We demonstrate this below from the text.



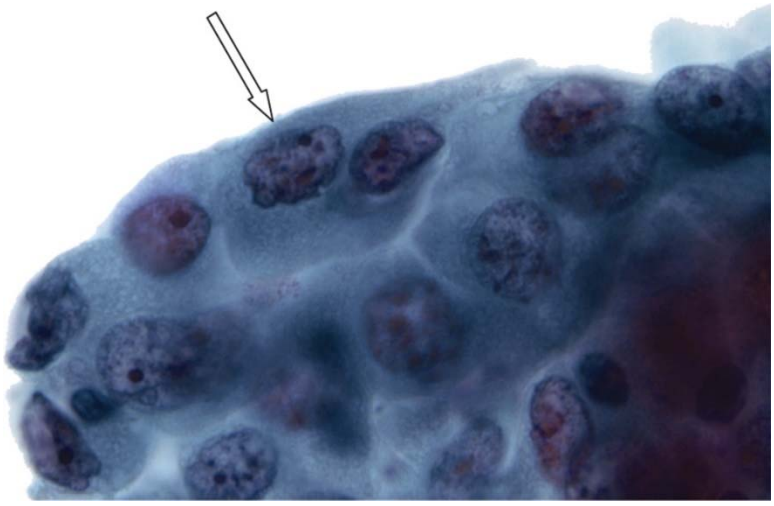
As with each of these characteristics we would like to seek someone to one relationship between this morphological change and the underlying genetic processes.

### *3.2.2 Nuclear Irregularity*

The nucleus may look rippled, or as noted below "raisin like". The question is; why? The internals of the nucleus are complex and generally consist of dormant chromosomes. This rippling is a significant and quite obvious variant. What is the cause, since we know that one would expect an even expansion of the nucleus and the structure has no clear underlying mechanism which has been identified. The authors note:

*The nuclear membranes of papillary carcinoma show irregularities of varying degrees. Most nuclei are oval with smooth, regular margins. However, it is not uncommon to find nuclei with strikingly irregular nuclear membranes, resulting in a "crumpled paper" or "raisin-like" appearance. One manifestation of this nuclear membrane irregularity is the nuclear groove. The nuclear grooves seen in papillary carcinoma have been given a variety of names, including linear chromatin ridge, chromatin band, nuclear folds, nuclear crease, or more accurately invaginations.*

Note the effect shown below.

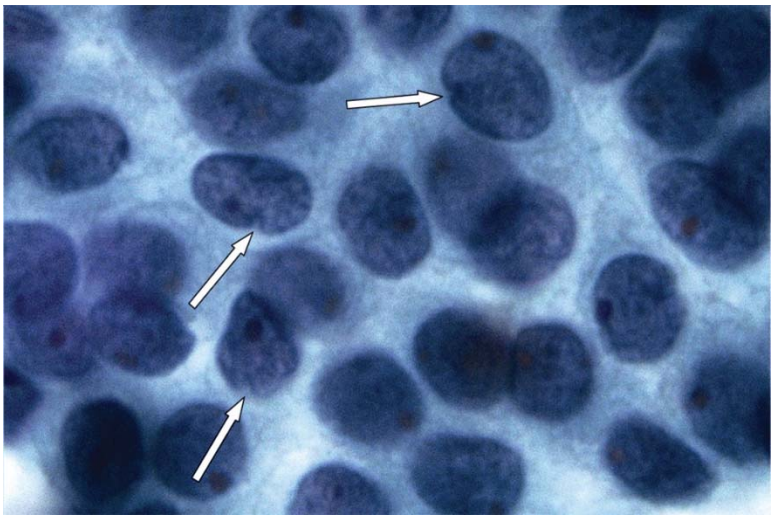


This "raisin" like effect of the nuclear wall appears to be pathognomonic. The question is: what are the underlying physical reasons and causes and what are the genetic factor, if any, yielding them?

### *3.2.3 Grooves*

As we noted above, a surface rippling is evident. Likewise, a groove or notch is also. The rippling is small scale and uniform and the groove is large scale and localized. The authors note:

*Nuclear grooves actually reflect an invagination of the nuclear membrane that runs parallel to the long axis of the elongated nucleus. Many grooves are fine and difficult to resolve by light microscopy. When well oriented and well resolved, one can appreciate that the groove is composed of two parallel lines representing the edges of the nuclear groove where the membrane has invaginated. If the orientation of the nucleus is such as to view the groove on edge, the invagination may be appreciated as a "notch".*

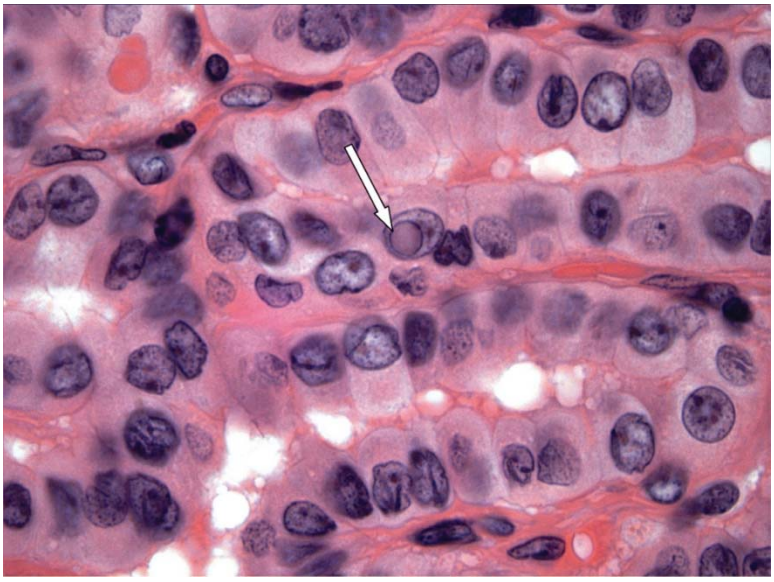


The challenging question is, as with all of these, what causes the grooves? What specific genetic control mechanism is at play?

### *3.2.4 Pseudo Inclusion*

Pseudo inclusions are intranuclear artifacts whose source is as of yet unknown. The discussion on these is as follows:

*When carried to an extreme and reflecting profound nuclear skeletal derangement, invagination of the nuclear membrane produces intranuclear cytoplasmic pseudoinclusions shortened to "intranuclear inclusions." Intranuclear inclusions have a high specificity for papillary carcinoma, although not 100%. However, to have this degree of specificity, the object in question must be an intranuclear inclusion and not a mimic such as an area of chromatin pallor. Thus to be considered an intranuclear inclusion, the object must fulfil four criteria. These criteria have been derived to achieve highly specific cytologic diagnoses but are equally applicable to histologic preparations*

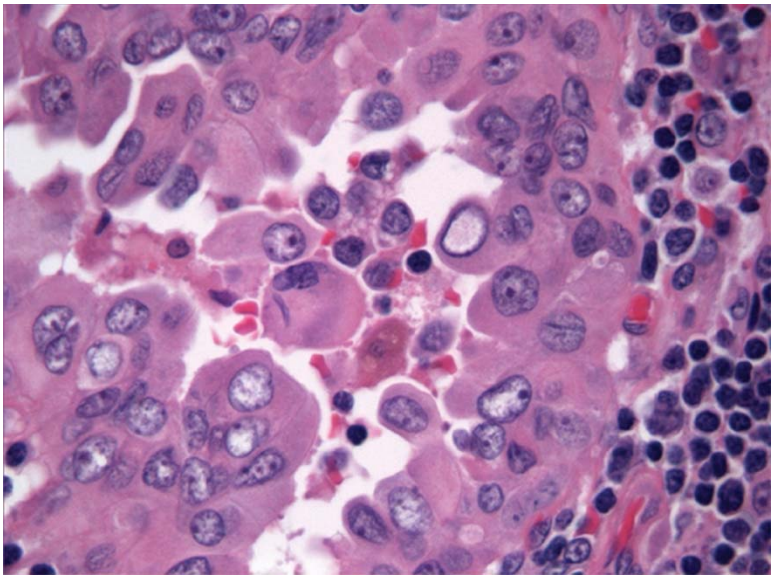


*The chromatin of neoplastic thyroid cells is altered and frequently becomes more pale and granular (powdery) in comparison to resting thyroid cells. Whereas chromatin changes are seen in many different thyroid neoplasms and are relatively nonspecific, the chromatin structure in papillary carcinoma is predisposed to the development of peripheral margination. As a result, the center of the nucleus develops a "ground-glass" appearance or, when pronounced, appears to be optically clear.*

The next artifact classed with inclusions is the "Orphan Annie Eye" inclusion as noted below:

*The chromatin is pushed to the edge of the nucleus, and the central clearing with thickened outline of the nucleus yield an appearance that resembles the large oval eyes of the cartoon character Orphan Annie, hence the term* ***"Orphan Annie-eye nuclei"****.*

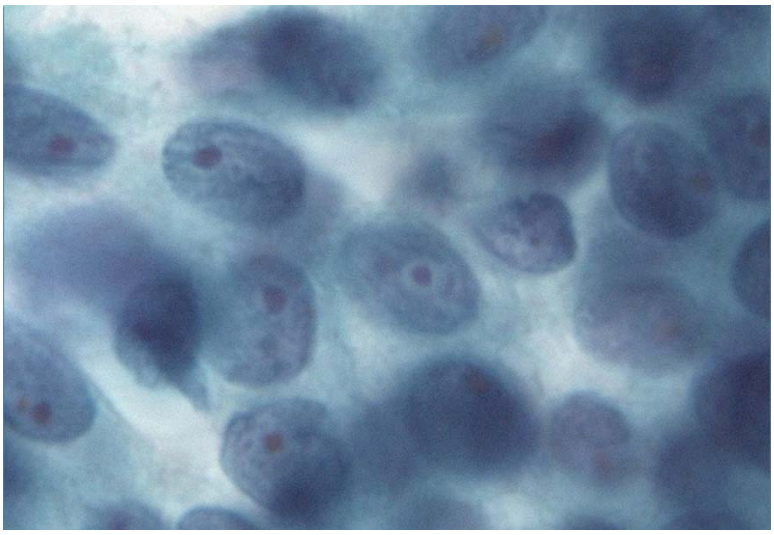
*This is a fixation artifact seen in formalin-fixed tissue and is not typically evident in frozen sections. It should be noted that the occurrence of optically clear nuclei is variable and influenced by the fixation conditions and has been reported in lesions other than papillary carcinoma. Optically clear nuclei are not described in routinely processed FNA of papillary carcinoma. However, it has been reported that optically clear nuclei can be induced in direct smears of the FNA specimens if the smears are first air-dried and then rehydrated followed by staining with a modified Pap stain.*



The question again here is just what is the source and mechanism associated with this artifact? The authors continue:

*The nucleoli of thyroid neoplasms are increased in prominence in comparison to resting follicular epithelium. The nucleoli do not take on the size and intensity of those seen in adenocarcinomas that arise elsewhere in the body but are instead seen as one to three micronucleoli, positioned toward the nuclear membrane. As usual, nucleolar feature is the development of "bare nucleoli" in which the chromatin surrounding the nucleolus is cleared, giving the appearance of the nucleolus residing within a hole.*

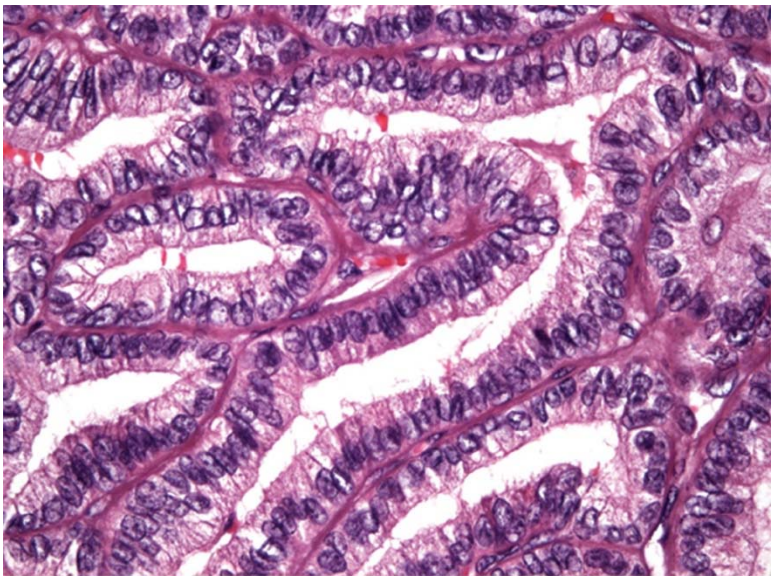
We demonstrate this below. The nucleolus is a significant player in many carcinomas as we have noted previously.



We understand what is in the nucleolus but we do not fully understand the expansion phenomenon. Genetically the nucleolus is focused on producing ribosomes and one would think its internal size was somewhat contained.

### *3.2.5 Tall Cell*

Tall cells, namely cells longer than wider as shown below are considered pathognomonic. Again we would ask; what is it that causes this extensive cellular morphological change?



## 3.3 GENOMICS

From Wells and Santoro we have the chart below which depicts the genetic disturbances for various thyroid cancers. The list is for point mutations and fusions. One interesting fact is that there are many thyroid cancers for which there are none of the mutations or fusions. One may ask; are there none at all or are we missing a significant number of genes yet to be determined.

The second questions would be; for these genes and for any yet to be determined, what are the dynamics related to cell morphology?

| | | | FA | HCA | NIFTP | FTC | HCC | PTC | PDTC | ATC | MTC[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Point mutations and indels | | *BRAF* V600E, % | | | | | 0 | 40-45 | 5-30 | 10-45 | <5 |
| | | *RAS*, % | 20-30 | 10-20 | 30-40 | 40-50 | 10-20 | 20 | 20-40 | 20-40 | 10-15 |
| | | *EIF1AX*, % | 10-20 | 10-15 | 5-10 | 10-15 | 10-15 | <5 | 10 | 10 | |
| | | *PTEN*, % | 10-15 | | -5 | 10-15 | 10-15 | <3 | 5-20 | 10-15 | |
| | | *DICER1*, % | 10-15 | | -5 | 10-15 | | <5 | | | |
| | | P53, % | | | | <10 | 15-20 | <5 | 10-30 | 50-70 | <5 |
| | | *TERT*, % | | | | 15 | 10-20 | 5-10 | 30-50 | 70 | |
| | | *PIK3CA*, % | <5 | | | <5 | | <5 | 5-20 | 5-18 | |
| | | *AKT1*, % | | | | | | | <5 | <5 | |
| | | RET, % | | | | | | | | | 40-50 |
| Large-scale alterations | Fusions | *RET/PTC*, % | | | | | | 5-10 | <5 | <1 | |
| | | *PPARG*, % | 5-10 | | 20-30 | 10-20 | | <5 | 5-7 | <1 | |
| | | *NTRK1/3*, % | | | | | | <5 | 1-5 | | |
| | | *ALK*, % | | | | | | <5 | 5-10 | <5 | 2 |
| | | *THADA*, % | 5-10 | | 20-30 | <5 | | 5 | | | |

## 3.4 CYTOLOGY RECAPITULATES GENOLOGY

The old adage, "Ontogeny recapitulates Phylogeny", or as has been said, "what comes first; the chicken or the egg", may apply here but in a different manner. Here we examine cytology, the characteristics of a cell, one in a malignant state. We look at its form, shape, structure, and then we ask what genes caused this.

| Cytological Character | Nexus | Genomic Defect |
|---|---|---|
| **Nuclear Enlargement** | | BRAF V600E |
| **Nuclear Irregularity** | | RAS |
| **Grooves** | | EIF1AX |
| **Pseudo Inclusions** | | PTEN |
| **Tall Cells** | | DICER1 |
| **EMT** | | P53 |
| **Other** | | TERT |
| | | PIK3CA |
| | | AKT1 |

From Garraway and Lander we have the following list:

| ***Actions*** | ***Genes*** |
|---|---|
| RTK signaling | *EGFR*, *ERBB2*,› *MET*,› *ALK*,› *JAK2*, *RET*,› *ROS*,› *FGFR1*,› *FGFR2*, *PDGFRA*,› and *CRKL* |
| MAPK signaling (oncogenes) | *KRAS*,› *NRAS*,› *BRAF*, and *MAP2K1* |
| MAPK signaling (TSG) | *NF1*› |
| PI3K signaling (oncogenes) | *PIK3CA*, *AKT1*, and *AKT3* |
| PI3K signaling (TSG) | *PTEN*› and *PIK3R1* |
| Notch signaling (oncogene or TSG) | *NOTCH1*, *NOTCH2*, and *NOTCH3* |
| TOR signaling (TSG) | *STK11*,› *TSC1*,› and *TSC2*› |
| Wnt/b-catenin signaling (TSG) | *APC*› and *CTNNB1*› |
| TGF-b signaling (TSG) | *SMAD2*,› *SMAD4*,› and *TGFBR2* |
| NF-kB signaling (oncogene) | *MYD88* |
| Other signaling | *RAC1*, *RAC2*, *CDC42*, *KEAP1*, *MAP3K1*, *MAP2K4*, *ROBO1*, *ROBO2*, *SLIT2*, *SEMA3A*, *SEMA3E*, *ELMO1*, and *DOCK2* |
| Epigenetics DNA methylation | *DNMT3A* |
| Epigenetics DNA hydroxymethylation | *TET2* |
| Chromatin histone methyltransferases | *MLL*,› *MLL2*, *MLL3*, *EZH2*, *NSD1*, and *NSD3* |
| Chromatin histone demethylases | *JARID1A*, *UTX*, *KDM5A*, and *KDM5C* |
| Chromatin histone acetyltransferases | *CREBP* and *EP300* |
| Chromatin SWI/SNF complex | *SMARCA1*,› *SMARCA4*, *ARID1A*, *ARID2*, *ARID1B*, and *PBRM1* |
| Chromatin other | *CHD1*, *CHD2*, and *CHD4* |
| Transcription factor lineage dependency or oncogene | *MITF*, *NKX2-1*, *SOX-2*, *ERG*, *ETV1*, and *CDX2* |
| Transcription factor other | *MYC*,› *RUNX1*, *GATA3*, *FOXA1*, *NKX3.1*, *SOX9*, *NFE2L2*, and *MED12* |
| Splicing | *SF3B1*, *U2AF1*, *SFRS1*, *SFRS7*, *SF3A1*, *ZRSR2*, *SRSF2*, *U2AF2*, and *PRPF40B* |
| RNA abundance | *DIS3* |
| Translation/protein homeostasis/ubiquitination | *SPOP*, *FBXW7*,› *WWP1*,› *FAM46C*, and *XBP1* |
| Metabolism | *IDH1* and *IDH2* |
| Genome integrity | *TP53*,› *MDM2*, *MSH*,› *MLH*,› and *ATM*› |
| Telomere stability | *TERT* promoter mutations |
| Cell cycle (oncogene) | *CCND1*› and *CCNE1*› |
| Cell cycle (TSG) | *CDKN2A*,› *CDKN2B*,› and *CDKN1B* |
| Apoptosis regulation | *MCL1*, *BCL2A1*, and *BCL2L1* |

Thus we have some modest nexus between genes and morphology but the details are still lacking.

## 4 VEGF

The vascular endothelial growth factor is a protein which matches with cell receptors and allows for the growth of vascular elements. These vascular elements are essential for the rapidly growing and proliferating malignant cells. Takahashi and Shibuya have noted:

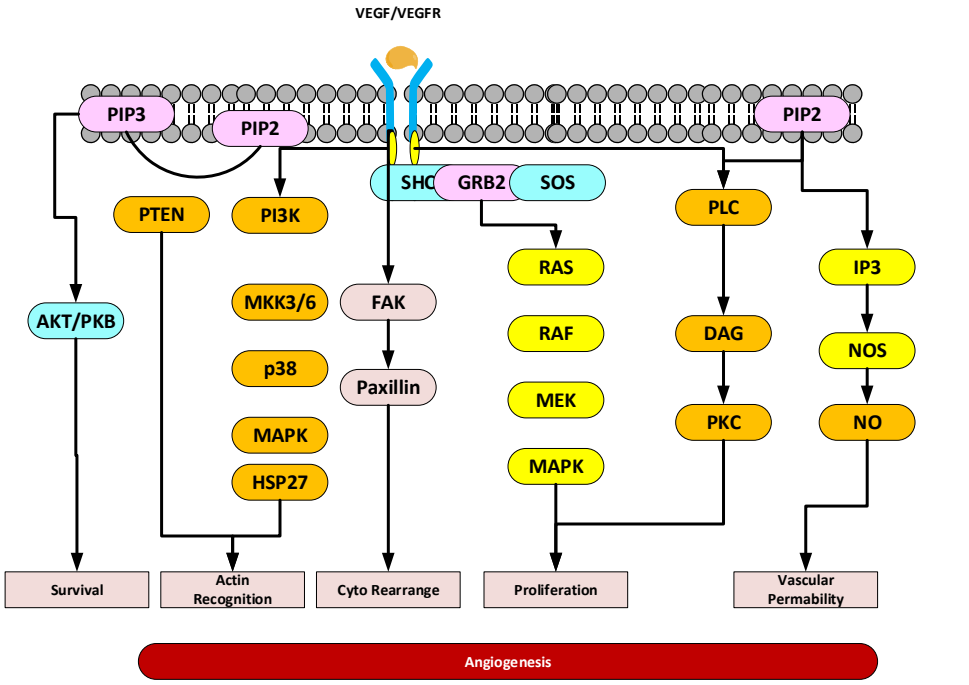
*The VEGF (vascular endothelial growth factor) family and its receptors are essential regulators of angiogenesis and vascular permeability. Currently, the VEGF family consists of VEGF-A, PlGF (placenta growth factor), VEGF-B, VEGF-C, VEGF-D, VEGF-E and snake venom VEGF. VEGF-A has at least nine subtypes due to the alternative splicing of a single gene. Although the VEGF165 isoform plays a central role in vascular development, recent studies have demonstrated that each VEGF isoform plays distinct roles in vascular patterning and arterial development.*

*VEGF-A binds to and activates two tyrosine kinase receptors, VEGFR (VEGF receptor)-1 and VEGFR-2. VEGFR-2 mediates most of the endothelial growth and survival signals, but VEGFR-1-mediated signalling plays important roles in pathological conditions such as cancer, ischaemia and inflammation.*

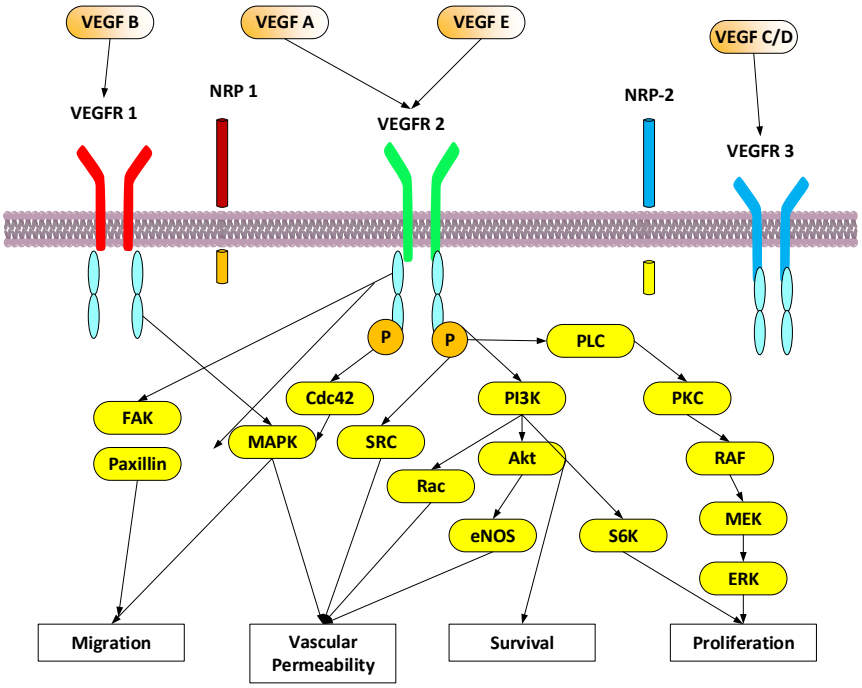
*In solid tumours, VEGF-A and its receptor are involved in carcinogenesis, invasion and distant metastasis as well as tumour angiogenesis. VEGF-A also has a neuroprotective effect on hypoxic motor neurons, and is a modifier of ALS (amyotrophic lateral sclerosis). Recent progress in the molecular and biological understanding of the VEGF/VEGFR system provides us with novel and promising therapeutic strategies and target proteins for overcoming a variety of diseases.*

The general pathways for VEGF are shown below:

http://www.sinobiological.com/VEGF-Signaling-a-1395.html?utm_source=journals&utm_medium=ntent&utm_campaign=VEGF-Signaling




In the above we focus on VEGF and its impact on angiogenesis. All of the subsets are representative elements for the ultimate ability to obtain vascularization. We now provide the authors, Takahashi and Shibuya, description of the fuller details of these pathways below:



The above is comparable to the first pathway diagram except it now details various VEGF elements which is more detail than the first pathway side.

As Witsch et al note:

*VEGFs regulate both vasculogenesis and angiogenesis.*

*The family consists of five glycoproteins, VEGFA (VEGF), VEGFB, VEGFC, VEGFD, and PlGF (placenta growth factor). In addition, alternative exon splicing generates four VEGF isoforms. Multiplicity also characterizes the respective surface receptors, co-receptors like neuropilins (NPs) and proteoglycans, as well as the downstream signaling pathways.*

*The VEGF family members bind to at least one of the three known VEGFRs, namely VEGFR-1 (FLT-1), VEGFR-2 (FLK-1 or KDR), and VEGFR-3 (FLT-4). VEGFA binds to VEGFR-1 and VEGFR-2, whereas VEGFB and PlGF bind exclusively to VEGFR-1. VEGFR-2 seems to mediate most known cellular responses to VEGF and has much higher intracellular signaling intermediates than VEGFR-1.*

*Unlike VEGFR-3, which is largely restricted to lymphatic endothelial cells, both VEGFR-1 and VEGFR-2 are expressed in vascular endothelial cells, as well as monocytes, macrophages (VEGFR-1), and hematopoietic stem cells (VEGFR-2). Importantly, expression of VEGFR-1 and VEGFR-2, as well as the co-receptors NP1 and NP2, has been detected on subsets of solid tumor cells, and according to a recent study activation of VEGFR-1 in breast cancer cells supports their growth and survival VEGFRs control angiogenesis by simultaneous signaling through several intermediates:*

*(i) cell proliferation and vasopermeability are stimulated by the protein kinase C pathway,*

*(ii) cell survival and proliferation by the AKT and MAPK pathway,*

*(iii) and cell migration results from signaling through SRC and Paxillin.*

*Other than the proangiogenic effects, VEGF exerts effects independent of vascular processes, such as autocrine effects on tumor cell function (i.e., survival, migration, invasion), immune suppression, and recruitment of bone marrow progenitors. The latter may dictate organ-specific tumor spread by homing to tumor-specific premetastatic sites and forming clusters that provide a permissive niche for incoming tumor cells*

We have demonstrated several of these pathways and receptors above.

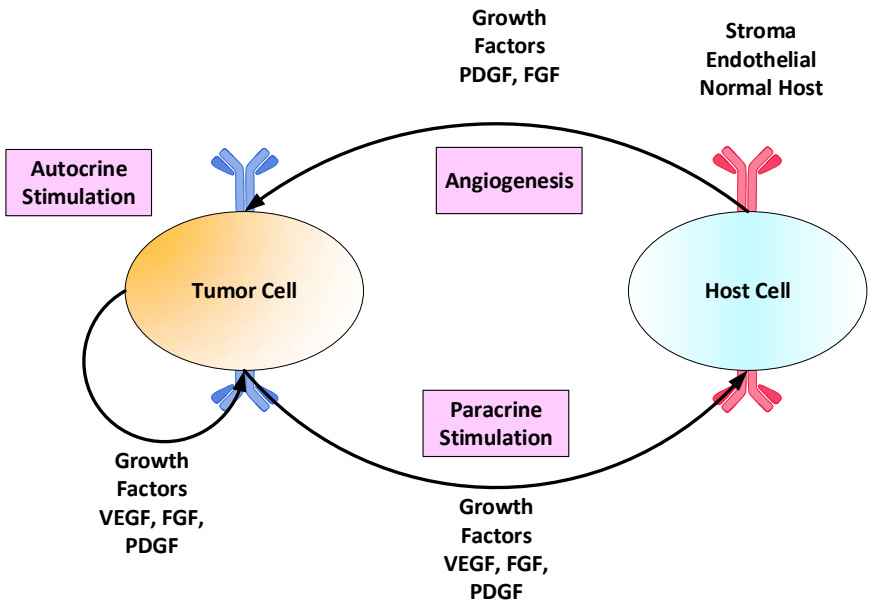
As Ferrara notes the possible targeting of VEGF for cancer therapeutics as below:

*Tumors require nutrients and oxygen in order to grow, and new blood vessels, formed by the process of angiogenesis, provide these substrates. The key mediator of angiogenesis is vascular endothelial growth factor (VEGF), which is induced by many characteristics of tumors, most importantly hypoxia.*

*Therefore, VEGF is an appealing target for anticancer therapeutics. In addition, VEGF is easy to access as it circulates in the blood and acts directly on endothelial cells. VEGF-mediated angiogenesis is rare in adult humans (except wound healing and female reproductive cycling), and so targeting the molecule should not affect other physiological processes. Tumor blood vessels, formed under the influence of VEGF, are disorganized, tortuous and leaky with high interstitial pressure, reducing access for chemotherapies.*

*Inhibiting VEGF would reduce the vessel abnormality and increase the permeability of the tumor to chemotherapies. Several approaches to targeting VEGF have been investigated. The most common strategies have been receptor-targeted molecules and VEGF-targeting molecules. The disadvantage of receptor-targeted approaches is that the VEGF receptors also bind different members of the VEGF super-family and affect systems other than angiogenesis.*

*The best-studied and most advanced approach to VEGF inhibition is the humanized monoclonal antibody bevacizumab…, which is the only anti-angiogenic agent approved for treatment of cancer*




Regarding the paper in discussion, Boos et al note:

*The VEGF gene underlies differential splicing into several isoforms that differ from each other regarding the inclusion of the exons 6 and 7 into the transcript of the gene, which is responsible for the binding of the protein to extracellular matrix heparin. This results in the diffusible isoform VEGF-A121 at one end of the spectrum and in the strongly heparin binding isoform VEGF-A189 on the other.*

*Thus, it can be assumed that VEGF protein expression measured by immunohistochemistry corresponds to the tissue bound isoforms but does not provide any information concerning the presence and amount of the soluble isoforms.* ***Since VEGF is secreted and high serum VEGF levels correlate with advanced tumor stages and lymph node metastases, it may thus well be possible that we observe a loss of VEGF expression on the immunohistochemical level in certain tumors because all VEGF has been secreted into the blood stream.***

***This would explain the high VEGF levels in serum and decreased VEGF expression by immunohistochemistry in patients who nevertheless might respond to VEGF inhibitors.*** *Unfortunately, there is no study available correlating the immunohistochemical expression of VEGF with VEGF serum levels and in our patients, serum is not available in order to test this hypothesis.*

As noted in the Boos paper the miRNAs target these various VEGF elements and in so doing can dramatically impact the growth of the tumor. Vascularization is a critical step in the progression from a local cancer growth to a metastatic condition. In addition as we have noted elsewhere the development of exosomes with specific miRNAs may be a significant element in metastasis and especially via the VEGF interaction. The above reference shows that VEGF serum levels are high in conjunction with the exosome flows of miRNAs.

## 5 PTEN

PTEN is a well know player in a variety of malignancies. For example, it has been found to be a key element in prostate cancer and in turn other malignancies. It acts as a critical control element in controlling pathways. As Canero notes:

*PKB/AKT constitutes an important pathway that regulates the signaling of multiple essential biological processes. PTEN is a dual protein/lipid phosphatase whose main substrate is phosphatidyl-inositol, triphosphate (PIP3), the product of PI3K. Increases in PIP3 result in the recruitment of PDK1 and AKT to the membrane where they are activated. Furthermore, PI3K can be activated by direct binding to oncogenic Ras proteins. Many components of this pathway have been described as genetically altered in cancer.*

***PTEN activity is lost by mutations, deletions or promoter methylation at high frequency in many primary and metastatic human cancers, and some germline mutations of PTEN are found in several familial cancer predisposition syndromes***.

*Activating mutations of PI3K occur in human tumors and confer tumorigenic properties to cells in culture. Taken together, this evidence indicates that the AKT pathway is a promising potential target for cancer chemotherapy. Indeed, many companies and academic laboratories have initiated a variety of approaches to inhibit the pathway at different points. Essentially, PI3Ks, PDK1, AKT and mTOR are heavily targeted for therapy in different ways. These proteins are kinases, which are very "druggable" targets a priori, and, according to the "addiction hypothesis", cancer cells with the activated pathway will be more dependent on its activity for their survival.*

The above description also calls into question the epigenetic impact of methylation. Methylation can alter gene expression but in addition one must examine the histone impacts as well. What happens in the nucleus in terms of the genes being expressed is as critical as the pathways themselves.

PTEN has been understood to play a role in thyroid cancers. As Boos et al have noted:

*Altered PTEN expression plays an important role in human cancer development. Mutations of PTEN have been detected in various other types of human carcinoma including breast cancer, endometrial and thyroid carcinoma. Despite this well-established knowledge, the prognostic role of PTEN has to our knowledge not yet been systematically evaluated in a large cohort of PTC patients. As expected, we found a decreased relapse-free survival in the case of PTEN protein loss. Interestingly, this proved to be an adverse prognostic marker independent of TC morphology in a multivariate analysis. We found a loss of PTEN protein expression in more than 50% of cases in the ACO group.*

*Since only 2-5% of PTC harbors a PTEN mutation according to the TCGA dataset and other studies, its loss of protein expression cannot be explained by mutations alone. One reason might be the epigenetic post-translational inhibition/cleavage via the upregulated miRNAs identified.*

*Some of them like miR-17-5p or miR-222-3p are already experimentally verified targets of PTEN while for others this work still has to be done.*

*All 32 miRNAs that we report to potentially target PTEN are upregulated and may therefore very well be accountable for the loss of the observed PTEN protein expression. In addition, all of them are significantly associated with a decreased RFS themselves in a Kaplan-Meier analysis.*

Again, the presence of the miRNAs is critical in terms of gene activity.

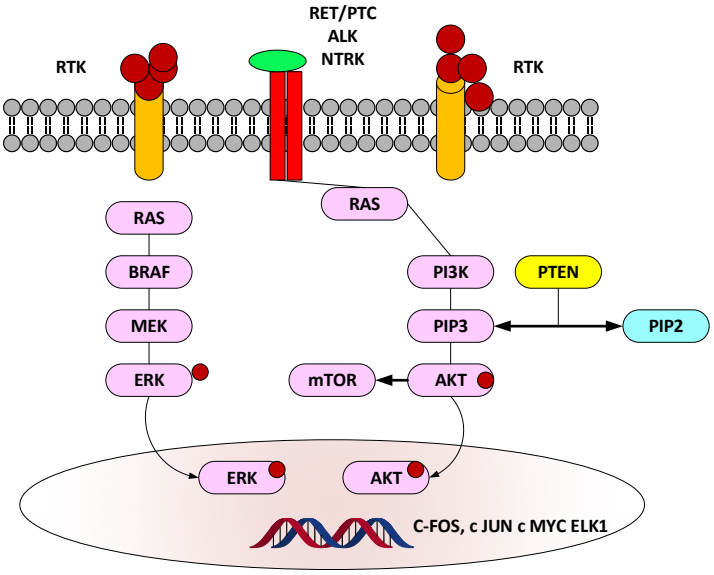
Now additional observations of PTEN in thyroid cancers are as Ringel et al note:

***Enhanced activation of Akt occurs in Cowden's disease, an inherited syndrome of follicular thyroid, breast, colon, and skin tumors, via inactivation of its regulatory protein, PTEN. Whereas PTEN inactivation is uncommon in sporadic thyroid cancer, activation of growth factor pathways that signal through Akt is frequently identified.***

*We hypothesized that Akt overactivation could be a common finding in sporadic thyroid cancer and might be important in thyroid cancer biology. We examined thyroid cancer cells lines and benign and malignant thyroid tissue for total Akt activation and isoform-specific Akt expression. In thyroid cancer cells, Akt 1, 2, and 3 proteins were expressed, total Akt was activated by insulin phosphatidylinositol 3-kinase, and inhibition of phosphatidylinositol 3-kinase reduced cell viability. In human thyroid tissue, increased levels of phosphorylated total Akt were identified in follicular but not papillary cancers compared with normal tissue.*

*Levels of Akt 1 and 2 proteins and Akt 2 RNA were elevated only in the follicular cancers. In paired samples, Akt 1, 2, 3, and phospho-Akt levels were higher in five of six cancers, including three of three follicular cancers. These data suggest that Akt activation may play a role in the pathogenesis or progression of sporadic thyroid cancer.*

From Wei et al we have the description of the PTN functions in TC as shown below.




Wei et al, **Targeting autophagy in thyroid cancers**

## 6 MIRNAS

We have examined miRNAs in multiple previous reports[6]. There are some 35,000 recognized miRNAs of which 2,000 play known roles in humans. As Hammond noted (2015):

*The discovery of the first microRNA (miRNA) over 20 years ago has ushered in a new era in molecular biology. There are now over 2000 miRNAs that have been discovered in humans and it is believed that they collectively regulate one third of the genes in the genome. miRNAs have been linked to many human diseases and are being pursued as clinical diagnostics and as therapeutic targets. This review presents an overview of the miRNA pathway, including biogenesis routes, biological roles, and clinical approaches.*

Depending on the source, the number is near or over 2000[7]. In the Boos et al paper under discussion, there are some 149 miRNAs, a significant proportion of the total 2000 known. Just what these miRNAs do is uncertain and furthermore as we have noted elsewhere they may have effects outside the initial cell[8].

In the paper by He et al they have noted:

*Apart from alterations in the RET/PTC-RAS-BRAF pathway, comparatively little is known about the genetics of papillary thyroid carcinoma (PTC). We show that numerous microRNAs (miRNAs) are transcriptionally up-regulated in PTC tumors compared with unaffected thyroid tissue. A set of five miRNAs, including the three most up-regulated ones (miR-221, -222, and -146), distinguished unequivocally between PTC and normal thyroid. Additionally, miR- 221 was up-regulated in unaffected thyroid tissue in several PTC patients, presumably an early event in carcinogenesis.*

*Tumors in which the up-regulation (11- to 19-fold) of miR-221, -222, and -146 was strongest showed dramatic loss of KIT transcript and Kit protein. In 5 of 10 such cases, this down expression was associated with germline single-nucleotide changes in the two recognition sequences in KIT for these miRNAs. We conclude that up-regulation of several miRs and regulation of KIT are involved in PTC pathogenesis, and that sequence changes in genes targeted by miRNAs can contribute to their regulation.*

They continue by observing:

*It is believed that miRNAs interact with target genes at specific sites by inducing cleavage of the targeted message or by inhibiting translation. Therefore, one might predict that an overexpressed miRNA would be associated with down-expression of its targets at the transcript*

[6] https://www.researchgate.net/publication/325106051_mi_RNA_and_Melanoma and https://www.researchgate.net/publication/331318495_Exosomes_and_Cancer

[7] http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsa

[8] https://www.researchgate.net/publication/331318495_Exosomes_and_Cancer

*or protein level. We examined the gene expression level of the putative targets of miR-221, -222, and -146 in the same set of RNA samples. This analysis was done first by evaluating genomewide gene expression levels in PTC tumors and matched normal thyroid tissues by using the Affymetrix chip. Significance analysis of microarray analysis revealed a list of genes differentially expressed (unpublished data). Most of these genes showed expression behaviors consistent with our previously published data. For example, overexpression of the CITED1 gene was found in all PTC tumors tested and was confirmed by semiquantitative RT-PCR*

Fundamentally the 149 putative miRNAs previously discussed need to be understood in terms of their functionality not just their presence. For example; are they functioning in a manner to alter the cell, nucleus, nucleolus and the like which one would observe cytologically?

As Rappa et al have just noted:

*Since the EV membrane protects RNA from blood-borne RNases and EV-associated RNA is generally free of endogenous RNA contaminants such as ribosomal RNA, EVs provide a more consistent source of RNA for disease biomarker detection compared with cellular or free plasma RNA. Interestingly, EV-associated miRNAs remain stable for years when EVs are stored at -20 °C.*

*The presence of functional RNA in EVs was first described in 2006 for murine stem cell-derived EVs and in 2007 for murine mast cell-derived EVs taken up by human mast cells. In ovarian cancer, a specific exosomal signature consisting of 8 miRNAs has been proposed as surrogate diagnostic for cancer screening in asymptomatic subjects. Another study on EVs of patients with melanoma found a correlation between down-regulation of circulating miR-125b and disease progression.*

*The group of miR-1246, miR-3976, miR-4644, and miR-4306 were up-regulated in EVs from 83% of pancreatic adenocarcinoma patients … discovered that miR-21 and miR-4257 expression in plasma EVs have potential as predictive biomarkers of recurrence in NSCLC patients. Similarly, circulating EV-associated miR-125a-3p in early-stage colon carcinoma, as well as miR-320, miR-574-3p, and RNU6-1 in glioblastoma multiforme, have been proposed as diagnostic biomarkers for early detection and monitoring of these specific types of cancer. A comprehensive list of EV-associated miRNAs is available in the miRandola database (http://mirandola.iit.cnr.it). Several studies on esophageal cancer, prostate cancer, and meningioma confirmed the power of EV-associated RNA in cancer diagnosis.*

The issue of EV has been examined previously[9]. However, the nexus between EVs and miRNAs seem to be stronger as they are studied. They continue:

*Moreover, specific alterations of cellular miRNA expression profile have been reported in thyroid carcinoma, indicating the possibility that some of these miRNAs, contained in EVs, may be employed as circulating biomarkers. miRNAs in the circulation have been analyzed as potential biomarkers of recurrence in PTC.*

[9] https://www.researchgate.net/publication/331318495_Exosomes_and_Cancer

*In many cases in which serum thyroglobulin measurements are difficult to interpret, the analysis of changes in circulating levels of miR-146a-5p and miR-221-3p in PTC patients indicate a good correlation with the American Thyroid Association (ATA)-defined response to therapy classes. …*

*that serum levels of miR-146a-5p and miR-221-3p could be used as complementary biomarkers for the early non-invasive detection of persistent PTC. The association between high circulating levels of miR-146b, miR-222, miR-221, and follicular thyroid proliferation has recently been described. Two miRNAs (miR-95, miR-190) were differently expressed in serum of PTC patients. In particular, miR-190 was up-regulated whereas miR-95 was down-regulated, which in combination can be used for the differential diagnosis of thyroid nodules.*

*Other studies have shown that the circulating levels of miR-146b-5p, miR-221-3p, miR-222-3p, and miR-146a-5p were reduced upon tumor excision. The up-regulated expression of miR-146b-5p, miR-221-3p, and miR-222-3p in the circulation of patients with thyroid cancer has also been demonstrated in PTC, as well as in anaplastic and follicular thyroid carcinoma Also, … found that plasma exosomal miR-21 and miR-181a differentiate follicular from PTC.*

The impact of miRNAs is expansive. In the brief discussion above we note a multiplicity of these small RNA units. Just how they modify what needs further examination. Just a listing of miRNA presence does not describe what they are doing in the target malignancy. Yet their presence is a reasonable marker.

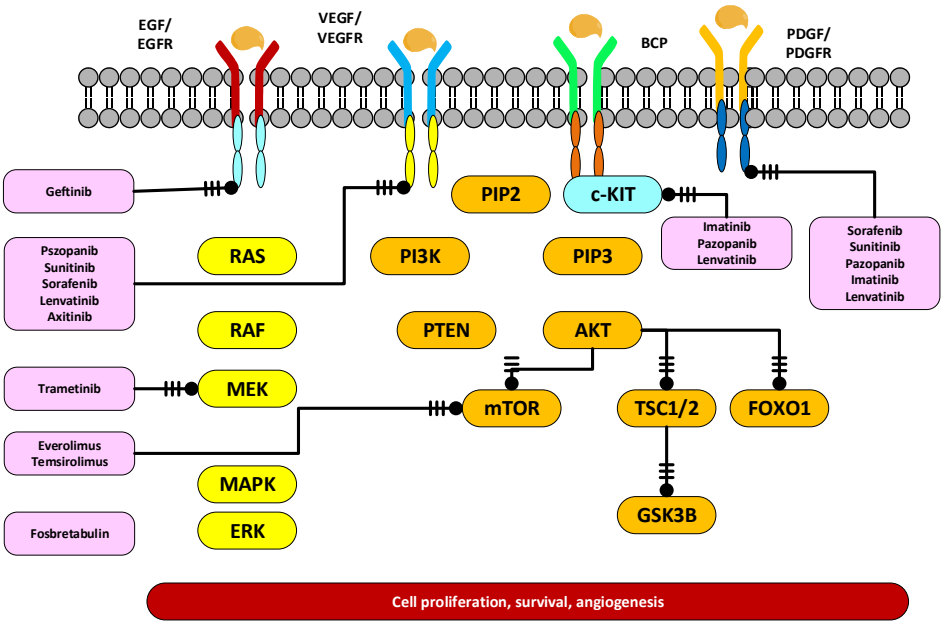
# 7 OBSERVATION

Understanding the morphological appearance of cells, singly and as a collection is the fundamental basis of cytology and histology. Historically examining cells microscopically was a process of understanding normal cell structure and then cataloging aberrant cells structures from a collection of malignant states. Much of this was accomplished prior to any understanding of the genomics of cancers.

In a sense this process was akin to the systematics of plants before genomic analysis, namely branching, inflorescence, root structure and many of the other Linnaean metrics to assign plants to families, genus and species. But with the advent of genomics, many plants got moved about. Our understanding of plants is changing as we move from phenotype to genotype. It will be interesting is we will see as much in human pathology.

## 7.1 THERAPEUTICS

There are a wide variety of therapeutics available. The chart below are a list and actions of some tried for anaplastic thyroid cancer. Besides these there is a new set of immunotherapeutic ones as well.




A Systematic Review of Phase II Targeted Therapy Clinical Trials in Anaplastic Thyroid Cancer Ljubas et al

## 7.2 SYSTEMS OF CELLS

The cells in any organ and in turn in any malignant growth appear to be a system of cells where there is an expression of multiple genes, namely in RNA of various types. The aggregate may

most likely be an integrated system, where they assembly is a non-random collage of cells, operating in some synergistic manner. Thus when, for example, we see a papillary structure we see the cells winding back and forth creating the papilla, and most likely there is some underlying differential gene expression creating this system. We can observe this but fail as of yet to explain why this is happening. We know that cell assembly is controlled by E-cadherin, and we know the micro-dynamics of the E-cadherin process. We know that EMT occurs when we lose E-cadherin. But this may or may not be part of the explanation for the papilla. The explanation may be dependent upon the extracellular matrix as well as surround cells.

## 7.3 LEVELS OF CONTROL

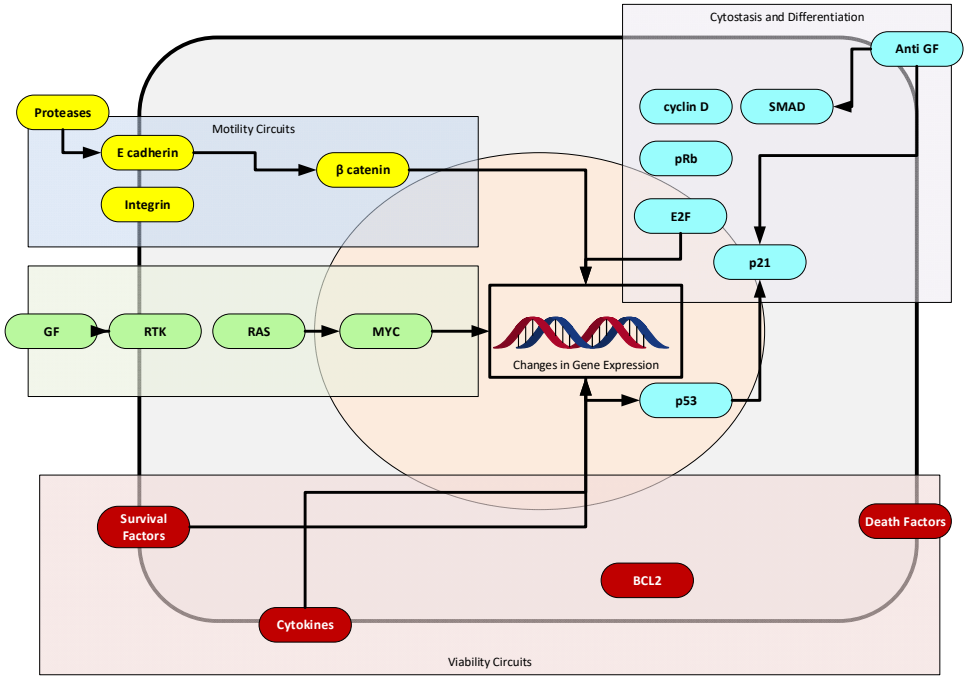
If we are to seek a way to control and manage cancers, we often seek certain ways to access, identify, and modify the cancer cells. We may ask; what makes a cancer cell different and how can we use that to identify it. Cytologically we have had almost a century and a half of significant work. We have looked at health cells and then at diseased cells. We look for markers reflecting the differences and then we record those difference markers pathognomonic for the suspected disease. Now with genetic markers we have putatively a cause and effect nexus. The loss of a PTEN results in proliferation. Thus, PTEN is pathognomonic. Or so we convince ourselves.

## 7.4 ARE PATHWAYS DISPOSITIVE?

A great deal of the current genomic analysis focuses on pathways and their ultimate control of the factors we know as proliferation, growth, and EMT. On the other extreme much of classic pathological examination is looking at cell shapes and aggregations and proliferations. The nexus between the two as cause and effect, namely in the context to shape is missing. Currently pathologists use both classic approaches as regards to morphology and they may also employ genomic testing. These two procedures are done as if they were separate and disconnected metrics. We do know that BRAF V600 is a marker for many malignancies, and we do have some modicum of understanding of its impact on E-cadherin and thus cell aggregation. But most of these linkages are haphazard and anecdotal.

Perhaps if we had a better understanding of the two, histology and genomics, we could rely on the genomics as being dispositive.

The issue we have been discussing herein is the impact of miRNAs on cellular structure and in turn on pathways. All too often we see analyses with pathways as if they were not impacted by miRNAs. The paradigm should be enhanced to include those effects as well. We have hundreds if not more pathways, but for the most part miRNAs are left as an afterthought. Thus, a dispositive pathway should in some sense include miRNA impacts.


Cytostasis and Differentiation
Anti GF
cyclin D
SMAD
pRb
E2F
p21
Proteases
Motility Circuits
E cadherin
β catenin
Integrin
GF
RTK
RAS
MYC
Changes in Gene Expression
p53
Survival Factors
Death Factors
BCL2
Cytokines
Viability Circuits

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