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The Evolving Role of Tissue Biospecimens in the Treatment of Cancer

Wilfrido D. Mojica
University at Buffalo, State University of New York
United States of America

1. Introduction

The field of surgical pathology is diagnostic in nature and since its inception, has always supported the treatment of cancer. The manner by which tissues are processed may vary between institutions, but the vast majority utilize formaldehyde as the fixative of choice. This fixative allows for excellent architectural tissue preservation thereby enabling optimal microscopic examination, the foundation from which surgical pathology is based upon. From fixed tissue, cancers have been categorized, sub-typed and features enumerated to help elucidate the differences that exist between them. Classification schemes that include grading and staging systems have been incorporated in their examination and, when coupled with the patient's clinical data, have been useful in prognostication and guidance of follow-up treatment. The development of immunohistochemistry has only served to further ingrain the importance of proper tissue fixation with cancer sample preparation. Yet, despite providing the basis for these invaluable contributions, the approach of fixing tissue biospecimens with formaldehyde has, in recent years, undergone criticism as being a suboptimal means of preservation when molecular analysis is desired from these specimens. Added to this are other pressures to develop novel approaches by which tissue biospecimens can be interrogated. Within the field of surgical pathology, it is becoming recognized that various pre and post fixation factors may contribute to negatively impact the overall integrity of the tissue biospecimen. Outside surgical pathology, the continually decreasing size of core biopsy specimens are minimizing the amount of tissue present for architectural evaluation. In addition, newer treatments are continually being sought in order to further personalize cancer treatment based on the type of tumor present in the tissue specimen. These forces are necessitating a re-evaluation of the entire established process of fixation, and whether alternative methods may exist or be developed that may be more amenable for both diagnosis and biospecimen integrity. In this chapter, the central role of tissue biospecimens for the support and guidance of cancer care are discussed, with an examination on how newer technologies and minimally invasive approaches will change the landscape by which these specimens will be processed.

2. The history of tissue biospecimens in cancer treatment

The examination of tissue through the microscope falls within the modern day purview of surgical pathology. Although this examination is now performed by pathologists, the

emerging field of surgical pathology in the 19th century began with surgeons. Before the widespread acknowledgement that microscopic examination of tissue was compulsory for an adequate diagnosis, surgeons were limited to the gross examination of specimens and their personal assessment and experience as to whether the extirpated specimen was benign or malignant (Gal, 2001). At that time, malignant tumors often ran their course, and their examination in the living patient limited to only observation at the gross, not microscopic level. In 1856 Rudolf Virchow published the first book on histopathology, introducing the world of microanatomy to the medical community. Observations from this book and those that followed slowly led to a newfound understanding that diseases of the human body could be correlated to findings at the cellular level. A number of discoveries and inventions in the 19th century also proved instrumental in providing the basis from which tissue could be reliably and widely examined by others under the microscope. These advances, like the invention of the microtome for cutting thin tissue sections and the discovery of natural and synthetic dyes like hematoxylin for the staining of nuclei and eosin for the cytoplasm of cells, allowed for the reproducible examination of tissue and the subsequent classification of organ structure. With these advancements, excised tissue was no longer discarded, but examined and documented. The first observations between malignant and benign tissues were recorded, with malignant cells described as being different in size and shape, not connected at their margins, possessing nuclei that varied in size and number and forming irregular edges at their juxtaposition with the adjacent normal tissue. In contrast, the cells of benign epithelial growths resembled each other in size and shape, had fairly homogenous appearing nuclei, were more cohesive along the edges of their cell borders and formed straighter lines (Rosai, 1997). With time, specimen processing and evaluation of the specimen became more standardized. Through correlation over time with the clinical outcome of the patient and with observations of similar tumors from other patients, these tissue biospecimens became the first to influence the treatment of cancer in patients. With improvements in surgical techniques, anesthesia and the application of antiseptic precautions, surgeons began to broaden the degree of surgical intervention they performed on patients. The development of the frozen section, wherein a portion of the specimen was excised and sent to the pathologist for evaluation intra-operatively, burgeoned and soon became integral to the cancer patient's care. The rapid freezing and hardening of the tissue specimen enabled the cutting of thin sections that could subsequently be stained and evaluated under the microscope. The surgeon now could know if the excised margins of a tumor specimen still had tumor cells in them, allowing for an intra-operative decision as to whether additional sections were needed or not. For certain other tumors, determining the depth of invasion through the frozen section became integral in planning out the remainder of the surgical plan for the cancer patient. The processing of the biospecimen would evolve to include frozen section evaluation on some, and fixation with paraffin embedding on the majority of excised tissue specimens sent to the pathology laboratory. Through the microscopic examination of all the subsequent slides, the diagnosis that is formulated by a pathologist is based on the resemblance and similarities to an archival history of previously diagnosed specimens that have been correlated with clinical outcome. This largely empiric system has served the medical community well, with the diagnostic information rendered by the pathologist enough to guide the cancer patient's care and estimate their prognosis (Kufe, 2003).

2.1 Early tools in the assessment of tissue and tumor biospecimens

Along with hematoxylin and eosin, a number of other stains were specifically developed to help identify cell structure and determine cellular function, but in the process aided to further classify tissues and categorize tumors. Their development was spurred by the fact that, despite the enumeration of histologic criteria published and disseminated about specific diseases and tumors, reproducibility among the growing numbers of physicians that practiced pathology remained a problem. In order to engender consensus, a number of special stains were created through experimentation that would eventually serve to better delineate cellular structure, content and properties. One of these stains, the Periodic Acid Schiff stain, allowed for the detection of the presence of carbohydrate macromolecules, a feature that would prove to be a useful ancillary aid to morphology in the differentiation between different types of tumors. For example, the identification of the Periodic Acid Schiff reaction in tumors known as small round blue cells can be helpful in leading to the correct diagnosis. Positive staining material in rhabdomyosarcomas, Ewing's sarcomas, malignant peripheral neuroectodermal tumors and germinomas can help distinguish them from other tumors with similar histologic features such as desmoplastic small round cell tumors, small cell mesothelioma, and a number of other tumors with small round blue cell morphology (Leuschner, 1996). This stain is particularly helpful in identifying Ewing's sarcoma, due to the fact that over 80% of these tumors have been reported to contain glycogen within their cytoplasm. Other special stains, like the Mucicarmine and Alcian blue stains, were originally used to help characterize cells that secreted mucin, aid in the early differential diagnosis of tumors and determine if they were useful in identification of tumor origin for metastatic tumors (Johnson, 1963). These stains have persevered and have modern day utility in defining poorly differentiated tumors as being or not being adenocarcinoma and in distinguishing adenocarcinomas from poorly differentiated squamous cell carcinomas in the evaluation of lung cancers for the former stain, and as an aid in the detection of intestinal metaplasia in the medical condition known as Barrett's esophagus for the latter stain (Wallace, 2009). Yet another special stain, the reticulin stain, showed initial promise for staining specific extracellular matrix constituents, namely the collagen type III fibers that comprise the stromal network in many different organs. It can be particularly prominent in the liver, as these fibers invest the hepatocytes that make up the hepatic plates, with normal hepatic plates being two cell layers thick or less. Reticulin stains help to define hepatic adenomas and hepatocellular carcinomas from normal liver, subtle changes not so obvious when examined by hematoxylin and eosin stains alone. In hepatic adenomas, they show an expansile growth pattern whereas in hepatocellular carcinoma they demonstrate an increase in trabecular thickness. To this day, the reticulin stain continues to be recommended as part of the evaluation of nodules within the liver (Lennerz et, 2009).

Later, the development of the electron microscope, with its ability to probe sub-cellular structure, proved a boon to tissue diagnostics as it related to cancer treatment. Delivery of the correct diagnosis enabled a treating physician the opportunity to give the most clinically relevant care. Electron microscopy allowed for the visualization of subcellular organelles that could otherwise not be discerned using traditional light microscopy. When tumor tissue was examined at the electron microscopy level, the presence or absence of organelles known to be specific to certain cell types enabled the identification of poorly differentiated tumors whose cell of origin could not, at the time, be properly classified at the morphologic level. The electron microscope provided more definitive characterization of tumor subtypes than

histology and special stains. In the case of Ewing's sarcoma, although it was known to be one of the tumors that possesses cytoplasmic glycogen, variability in the degree of differentiation could mean that their abundance may also be variable, and hence it was advised that the diagnosis of Ewing's sarcoma should not be based solely on the presence of glycogen detected by the Periodic Acid Schiff stain in tissue sections with the appropriate morphology. One factor may have played a role in this ambiguity, was the type of fixative being used. Eventually it was determined that the best fixative for detecting glycogen in tissue sections was alcohol, and that fixation in formaldehyde resulted in variable preservation of this macromolecule (Llombaart-Bosch, 1996). Additionally, it was also learned that specimens that were poorly fixed could lead to a complete absence of detectable glycogen. In these cases, ultrastructural evaluation would prove to be helpful in establishing the correct diagnosis. In the case of Ewing's sarcoma, examination at the ultrastructural level revealed the presence of two cell types, the primary cell referred to as the light or principal cell, and a secondary cell referred to as the dark cell. The principal cell was characterized as having homogeneously sized nuclei and ample cytoplasms, sparse numbers of organelles and abundant glycogen. The dark cells possessed elongated to ovoid nuclei with condensed chromatin, and likened to involuting principal cells. The identification of these cells have helped to characterize Ewing's sarcoma as different from other tumors possessing a similar histologic appearance, particularly olfactory neuroblastomas (Trump, 1983). Another instance where electron microscopy has impacted cancer care and proven integral to the proper identification of a tumor is in the case of poorly differentiated tumors of the pleural cavities. In these situations the differential diagnosis revolves between poorly differentiated adenocarcinoma versus mesothelioma. An incorrect diagnosis of adenocarcinoma, when in reality a tumor is a mesothelioma, can result in an expensive and time consuming work-up and legal issues. For quite a while, electron microscopy was considered the gold standard in the diagnosis of mesothelioma. The presence of long, slender, sinuous, branching and bushy microvilli found on the cell surface at the ultrastructural level were reported as being pathognomonic features for diagnosing mesothelioma (Velez, 2002). Taken altogether, the development of special stains and electron microscopy aided in leading to the further sub-classification of tumor tissue biospecimens. These tools were readily incorporated into the surgical pathology community and contributed to the early attempts to tailor patient cancer care. Despite the advances these tools brought to patient cancer care, their limitations would eventually become apparent with the development of a newer tool that would be introduced to the armamentarium of surgical pathology, immunohistochemistry.

2.2 Immunohistochemistry and the beginning of the end for empiric medicine

In the latter half of the 20th century the ability to exploit the specificity of the antibody-antigen reaction was successfully transferred from the experimental laboratory to clinical specimens. This application began with the immunofluoresence technique. However, the major liability with this approach was three-fold: the need for a specialized microscope with fluorescence capabilities; the pre-requisite for fresh frozen tissue samples; and poor morphologic resolution (Taylor, 1994). Over the period from the mid 1970's to the early 1990's these obstacles were eventually addressed with the development of alternative, non-fluoresence labels and the discovery of antigen retrieval. In the latter, the abolishment of the interfering formalin induced cross-links in fixed tissue specimens led to the widespread application of this technique, now called immunohistochemistry, to the vast archive of fixed tissue specimens banked in pathology departments. A period in the surgical pathology

community began anew, similar to the advent of special stains and electron microscopy eras, wherein panels of recently developed antibodies were tested against series of tissue and tumor specimens with the intent to identify and again better characterize human disease. This period, as with the introduction of all new tools in the fields of diagnostic pathology, was met with initial skepticism. However, one key difference that this new tool brought would emerge that would distinguish it from that of its' predecessors in the care of cancer patients. The previous tools only enabled the observation of cellular organelles and cytoplasmic or nuclear constituents. The technique of immunohistochemistry, with the specificity of the antibody-antigen relationship, allowed unprecedented access to the macromolecules integral to the functions of the cell, proteins. Through subsequent investigations of an assortment of proteins by a myriad of different investigators, the era of Personalized Medicine, in terms of its current day namesake, was unceremoniously ushered in. It now became possible to identify the presence or absence of proteins in specific cell types and tumors. In contrast to traditional empiric medicine, immunohistochemistry allowed for the identification of a specific target molecule in specific cell types. In empiric medicine, not all patients will respond to a specific drug based on the absence of the knowledge if the cancer cells in a patient contained those proteins acted upon by the drug, or had only low levels of those targeted proteins. With the advent of immunohistochemistry, proteins that were involved in or acted to drive the process of oncogenesis could now be identified. This identification allowed for rational drug treatment, with therapy based on the presence of a target protein or molecule in a cancer patient identified by immunohistochemistry on the tissue specimens. This approach represents the potential to significantly improve cancer care, taking into account the fact that the efficacy of pharmacotherapy in oncology is less than 50% (Jorgensen, 2009). In the very least, this approach will help eliminate the administration of certain therapeutic agents to those cancer patients who would not benefit from a drug, based again on the absence of the targeted molecule or protein in the patient's tissue specimen.

Possibly the first successful implementation of Personalized Medicine can be attributed to the steroid receptor estrogen in human breast cancers. The observations in preceding decades by physicians and scientists that the growth of certain reproductive organ related tumors appeared dependent on sex steroids led to further direct investigations. Eventually it was borne out that certain tumors, like those of the breast, possessed large numbers of the estrogen receptor and thus could be targeted for endocrine therapy. Later it became apparent that the amount of estrogen receptors in these tumors could be variable, and that patients with estrogen receptor positive tumors tended to have a better clinical course than those patients with estrogen receptor negative tumors. It thus became imperative to be able to determine the estrogen receptor status in these patient's tumors. The method that became the initial mainstay to assess estrogen receptor status was the steroid ligand binding assay and involved the homogenization of tumor tissue into a lysate that was then exposed to labeled estradiol. This assay however, lacked adequate specificity and sensitivity for the clinical setting. The major drawback of this assay was the fact that it was based on a tissue homogenate and was therefore without any correlative histologic picture. Thus the proportion of tumor cells to stromal cells, or the amount of necrosis present in the sample submitted could not be accounted for in the sample. Additionally, improper collection of the sample, that is, prolonged procurement time leading to artificial loss of this labile receptor, could bias the final results. With the concurrent progress in immunohistochemistry, the development of an antibody suitable to test on frozen, and then ultimately formalin fixed and paraffin embedded tissue became available that ultimately

showed suitable concordance with the steroid based assay (Ottestad, 1988). Immunohistochemistry, because it enabled visualization of the receptor status on glass slides, was relatively inexpensive compared to the ligand binding assay and could evaluate small tumors, gradually gained acceptance as a means to evaluate steroid receptor status in breast cancer patients. Currently, immunohistochemistry is the standard by which estrogen and progesterone receptor status are assessed. Their assessment in the breast cancer patient is integral in guiding that patient's direction of clinical care, as endocrine therapy has proven to be of benefit only to those that have tumors that are estrogen receptor positive. However, recent emphasis on the importance of proper tissue handling has been raised again bringing back into focus the importance of the tissue biospecimen's role in cancer care. A recent collaborative effort by the American Society of Clinical Oncology and College of American Pathologists has brought to light the startling finding that up to 20% of estrogen or progesterone receptor findings by immunohistochemistry may be inaccurate, either being falsely negative or falsely positive (Hammond, 2010). These findings were determined by taking the original results and testing the same tissue blocks at an experienced immunohistochemistry central laboratory for comparison. In order to rectify this problem, this collaborative group published recommendations for the optimal handling of extirpated breast cancer specimens that under present day methods of tissue processing, would result in reproducible inter-laboratory steroid receptor studies. In this instance, breast cancer tissue biospecimens play a continuing role in the guidance of clinical care and the quality assessment of diagnosis.

The story of the Her-2 gene and the development of the humanized monoclonal antibody trastuzumab represents a case wherein tissue biospecimens aided in the rapid identification and confirmation of a targeted cancer therapy. Through the use of tissue biospecimens, researchers were able to identify a subset of patients that overexpressed the gene product of the Her-2 gene. With clinical correlation, this subset was determined to be associated with a worse overall prognosis and a relative resistance to endocrine therapy (Press, 1993). But more importantly, the presence of this overexpressed protein meant that it defined a particular group of breast cancer patients who might benefit the greatest by the creation of an anticancer agent directed specifically at that amplified gene product. The biotechnology company Genetech eventually developed a recombinant monoclonal antibody that fit within a specific extracellular cleft and effectively abrogated any further tyrosine kinase activity. An antibody was also subsequently developed that could be used on tissue biospecimens to detect this proteins presence in breast cancer cells. Through the use of banked tissue, this antibody was tested on breast cancer tissue biospecimens, the results from which produced an FDA approved assay. The publication of these results led to the ability for pathology laboratories, community in addition to academic, to incorporate this immunostain into their diagnostic regimen. The broad use of this immunostain led to the findings that the prevalence of the HER2 gene amplification in the breast cancer population was consistently between 20 to 30 percent. Similar to estrogen and progesterone receptors, continued research in the field of biospecimen science led to the conclusion that approximately one fifth of all breast cancer tissue specimens immunostained for the Her-2 protein could be inaccurate (Wolff, 2007). A number of sources for variation were identified, ranging from preanalytic (time to fixation, method of tissue processing, time of fixation, type of fixation), analytic (assay validation, equipment calibration, use of standardized laboratory procedures, training and competency assessment of staff, type of antigen retrieval, test reagents, use of standardized control material, use of automated laboratory methods) and postanalytic (interpretation criteria, use of image analysis, reporting elements, quality

assurance procedures). Due to the number of sources of variation and the influence they could have on the tissue biospecimen, a number of situations were enumerated that were grounds to not use immunohistochemistry to assess Her-2 protein levels. These exclusionary criteria included the use of any fixatives other than formalin, needle biopsies fixed for less than 1 hour, excisional biopsies fixed for less than 6 hours and longer than 48 hours, core needle biopsies with edge or retraction artifact affecting the entire core or with crush artifact, tissues with strong membrane staining on internal normal ducts or lobules and tissues with controls that showed unexpected results. The initial and continued use of tissue biospecimens to assess Her-2 overexpression have been integral in creating appropriate standards aimed at establishing a uniform, reproducible result. In turn, these results continue to help further refine the clinical decision process in the care of breast cancer patients.

The use of tissue biospecimens also played a significant role in expanding the utility of certain drugs in the treatment of cancer. Imatinib mesylate (Gleevec), originally designed to target the chronic myeloid specific protein BCR-ABL, was later found to show some activity with another tyrosine kinase, notably the gene product KIT. Using tissue biospecimens, investigators were able to document that a relatively rare gastrointestinal tumor, called a Gastrointestinal Stromal and Tumor, expressed the KIT gene (Hirota, 1998). Treatment of Gastrointestinal Stromal patients with this therapeutic agent produced remarkable results. These previous examples illustrate how tissue biospecimens have helped shape the development of therapeutic agents for the treatment of cancer. A known protein that can be targeted is identified, a potentially therapeutic agent created or already is in existence, and assays developed that can identify those protein(s) on tissues. The changing paradigm is to treat a patient's tumor with an agent that is antagonistic to a protein or molecule that can be documented to be present in the tumor tissue. This is in contrast to treating the tumor based on a histologic classification and previous experience of response in a certain percentage of patient's with that tumor type. In this new era of Personalized Medicine, therapies will be given specifically to those individuals most apt to respond to them. Therefore, those who will benefit from a targeted therapy will be appropriately selected for treatment, and those who will not benefit will be treated by another regimen. Tissue biospecimens will continue to play a significant role in the new medical paradigm, however their traditional role may evolve. Whereas the current means of evaluating tissue biospecimens is after fixation and processing, with the end result a paraffin embedded specimen that is stained for visual examination either through traditional hematoxylin and eosin stains complimented by immunohistochemistry, tomorrow's biospecimen may be subjected to molecular assays. A representative scenario is the case of CD-117 positive tumors. Initially, Gastrointestinal Stromal Tumors were found to express this protein in abundance. By similar reasoning, it was assumed that other CD-117 positive tumors could also be treated by this tyrosine kinase inhibitor. A number of CD-117 positive tumors were rapidly identified that included colorectal cancers, renal cell carcinomas, thymic epithelial tumors, seminomas, Merkel cell cancers, endometrial stromal sarcoma and aggressive fibromatosis (Quek, 2009). However, the clinical community soon came to the realization after much scientific investigation that the underlying molecular alterations in Gastrointestinal Stromal Tumors, namely the gain of function mutations in the C-Kit gene in specific exons, were the underlying reason for their therapeutic responsiveness. Although other tumors may express the C-Kit gene product and can be readily identified as being CD-117 positive, without the specific mutations seen in GIST tumors, these other tumors turned out to be non-responsive to Gleevec administered

therapy. This example highlights how the oncoming molecular age in medicine will play a role in the analysis of biospecimens for the rational delivery of targeted therapy.

3. Achieving personalized medicine through improved diagnostics

Although conventional light microscopy has been the stalwart of diagnostic pathology for decades, it continues to have problems with reproducibility among members of its collegium. As a continually evolving field, newly described entities go through the academic rigors of debate before consensus is reached. This can take some time, with consensus established after many years. Several examples showcase the limitations of diagnosis based solely on morphology. In the colon, preneoplastic polyps have traditionally been classified as either hyperplastic or adenomatous. In the late 1990's, two new entities were described called the serrated adenoma and the admixed polyp. Despite a decade where these entities were studied and further described, some confusion still remains regarding their correct diagnosis. When specialist gastrointestinal pathologists were shown a number of cases of these two entities, only a moderate degree of concordance was noted, and only fair inter-observer agreement evident (Wong, 2009). When cells alone are examined, that is, in the form of a cytology specimen, molecular diagnosis can be an improvement. The detection of atypical cells in sputum cells prepared for cytologic examination for the screening of lung cancer is a proven method, but suffers from low sensitivity. The addition of genomic markers can raise the sensitivity of cancer detection to 86% and specificity to 93% (Jiang, 2010). When it comes to actual tumors, interobserver agreement has had a history of similar problems with at best, modest reproducibility. One of the more difficult tumors to classify are those epithelial tumors originating from the ovary. Through decades of examination, diagnostic reproducibility based on morphology has steadily increased (Kobel, 2010). Aiding this improvement has been immunohistochemistry and molecular analysis. There are now considered to be five major subtypes of ovarian carcinoma: high grade serous, clear cell, endometrioid, mucinous and low grade serous carcinoma. The importance of this new paradigm based classification again is treatment based, with high grade serous being responsive to neoadjuvant chemotherapy whereas the others are not. From continued research utilizing all available data from the patient, the clinical outcome and most importantly the tissue, consensus in diagnosis is being achieved with diagnostic algorithms to achieving reproducible results being reported (Kalloger, 2011).

These examples demonstrate the limitation of histologic diagnosis and the increasing role molecular analysis will play in identifying, classifying and prognosticating tumors. Assays that will be developed to achieve this will most likely be used to interrogate nucleic acids, whether DNA or RNA, but may also analyze proteins. The arrival of these assays may be tied directly to therapy and hence have been given the designation companion diagnostics (Papadopoulos, 2006). The implications of using these molecular approaches are that they may eventually yield more informative data than conventional hematoxylin and eosin stained microscopic slides, even when the tissue is further worked up with immunohistochemistry. The goal is to be able to identify subgroups within a given patient population whose histology may appear similar, but end up having a different clinical course or outcome. It is hoped that through the molecular evaluation of tissue specimens, molecular signatures can be identified and assays developed that can recognize and separate these subsets of patients from the general cancer population. A realistic future scenario where molecular studies may eventually take precedence over histology is with

Stage II and III adenocarcinoma of the colon. Although histologically these tumors may appear morphologically similar and stage similarly, it is known that a certain proportion of these patients do benefit from adjuvant chemotherapy whereas for the other proportion of this group it is unnecessary. The identification of a biomarker or biomarkers capable of clearly delineating these two subpopulations would save time, effort and benefit the patients having to be and those not having to be treated. Another reason molecular analysis may be more informative than traditional hematoxylin and eosin stained sections is its objective nature. There are a number of tissue types from which human bias has been known to exist. The classification of dysplasia in the oral-pharyngeal space has for years been known to suffer from inter- and even intraobserver variability (Karabulut, 1995 and Abbey, 1995). For gliomas of the brain, histologic classification, which guides subsequent therapy, suffers from interobserver variability (Kros, 2007).   Even with an updated classification scheme, diagnostic variability persists now due to the addition of newly added entities and variants (Tremblath, 2008). It would appear logical then, that another mechanism of diagnosing these types of tumors would be attempted. In fact, a recent study did find that molecular analysis did perform better than traditional histology, albeit in the realm of survival prognostication (Gravendeel, 2009).   Thus it appears that with the molecular era steadily encroaching the clinical realm, the dedication of a proportion of tissue from excised tumor biospecimens may be needed as part of the standard of care for the cancer patient. The tissue biospecimen will still be integral in patient care, but the manner by which it is examined will evolve.

4. Barriers to the molecular profiling of clinical specimens - formalin fixation

Several barriers exist that limit the application of molecular techniques to tissue biospecimens. Since the main goal of extirpated tissue is the establishment of a diagnosis, the priority for such specimens is the optimal preparation of a tissue section for morphologic evaluation. In order to do this, tissue preparation involves fixation and processing, followed by sectioning and staining for microscopic examination. In the vast majority of community and academic pathology departments, the fixation used is 10% neutral buffered formaldehyde. Whereas this fixative results in a reliable and reproducible end product for histologic examination, it has been shown to be detrimental to the recovery and examination of cellular molecules. Formaldehyde interacts with DNA to create hydroxymethyl groups, forms methylene bridges between amino acids, generates apurinic and apyrimidic sites that lead to highly unstable cyclic carboxonium ions that hydrolyze into 2-deoxy-D-ribose, and cause the slow hydrolysis of phosphodiester bonds that result in short chains of polydeoxyribose with intact pyrimidines. In short, the chemical reaction between formaldehyde and deoxyribose nucleic acids leads to the denaturation of these molecules as well as the formation of cross links with proteins. The result is the recovery of shortened segments or fragmented DNA and decreased recovery. For certain high throughput downstream molecular based assays, like array comparative genomic hybridization that are designed to evaluate the copy numbers of genes from the entire genome, the absence of gene segments may lead to erroneously biased conclusions relating to genetic deletions. Several reports have noted that DNA extracted from formalin fixed, paraffin embedded tissue blocks and run on an array comparative genomic hybridization platform, tended to yield data prone to spurious changes in genetic copy number in addition to copy number loss when compared to matched fresh frozen tissue (McSherry,

2007). To complicate matters worse, the presence of formaldehyde results in the presence of high background 'noise' on array comparative genomic hybridization data, making the determination of legitimate significant molecular alterations problematic (Johnson, 2006 and Mojica, 2008). At the nucleotide level, formaldehyde has been documented to result in random changes in amplified sequences relative to the original DNA sequence. Formalin fixation is thought to cause base damage, but the overall template can still be read by polymerases. When PCR is performed using *Taq* polymerase, errors in translesional synthesis can occur (Quach, 2004). Although this error rate is low, it can be a factor when small amounts of originating material are used. This has grave implications at the clinical level, as the reporting of non-existent mutations may severely impact a patient's care altering the treatment regimen from which the fixed tissue originated from.

RNA is another potential biomarker that can be assayed from tissue biospecimens, and in the past decade has already influenced the realm of pathologic diagnostics in the field of breast cancer. Through expression array analysis, breast cancers now are considered to comprise a number of subgroups (normal-like, luminal, basal-like, HER-2(+)) within the broad spectrum of what was originally only considered part of one entity, ductal adenocarcinoma. Work is currently progressing on finding the most appropriate therapeutic regimen for each subtype. Based on the clinical success of this molecular based classification, other tumors are being probed for their molecular signature. However, it must be noted that the original work that defined these breast cancer subtypes was based on fresh frozen material. Any subsequent discoveries on such tissue must be able to be translated to clinical material, which again routinely undergoes formalin fixation and processing. Similar to DNA, mRNA recovery from FFPE tissue is encumbered by the crosslinking of molecules and poor recovery (Cox, 2006). But since mRNA is a more labile molecule than DNA, degradation of the RNA molecule impacts the overall recovery more with significantly reduced quantities and poor quality obtained from fixed tissue specimens when compared to matched frozen material (Specht, 2001). This negatively impacts the consideration of using FFPE tissue biospecimens for gene expression on cancer samples. RNA extracted from FFPE tissue is partially degraded resulting in gene expression data with low signal intensity data. In one study, only a quarter of unselected samples that were FFPE provided enough starting material for subsequent gene expression assays (Penland, 2007). In another study, gene expression values differed by a value greater than two fold in almost 20% of the genes studied between matched FFPE and frozen tissue samples (Mojica, 2007). This rather significant discrepancy can be accounted for by either a decrease in gene expression due to degradation or an increase in gene expression due to changes in the tissue microenvironment and the cells subsequent reaction prior to fixation. The major implication however, in regards to human tissue that could be potentially assayed for mRNA expression levels that may influence clinical care, is to determine beforehand which mRNA transcripts are stable, and remain stable. It is these transcripts that would be of clinical value, as opposed to those more labile ones prone to either degradation or biased due to *ex vivo* conditions (Lee, 2005). This obviously would require an in depth investigation into which transcripts are most stable and least resistant to the external pressures associated with the tissue fixation process, so that those that are susceptible to degradation during the process of fixation are excluded from further consideration and studies (Opitz, 2010).

Proteins are the last major molecule to be mentioned here that are routinely assayed from clinical tissue biospecimens. The formalin-fixed, paraffin-embedded tissue specimens

routinely examined in surgical pathology departments currently undergo proteomic evaluation in the form of immunohistochemistry. However, this analysis is often limited to evaluating only one, or at most two proteins in the tissue section. When tissue biospecimens are exceedingly small, the tissue may get exhausted, limiting the number of proteins that can be examined through immunohistochemistry. An approach that may see increased use, especially when a number of proteins will need to be evaluated in a tissue biospecimen, is mass spectrometry. Currently, mass spectrometry is finding initial success as a diagnostic modality for amyloidosis, and its integration into other pathologic conditions most likely will soon follow suit. However, as with DNA and RNA, the analysis of the traditionally formalin-fixed, paraffin-embedded tissue biospecimen by mass spectrometry will present with problems once again associated with formaldehyde. The cross links formed in proteins increase the complexity of data analysis and peptide identification through the addition of 12 and 30 Dalton changes in the peptide mass (Metz, 2006). Adding to the complexity of this reaction is the finding that with time, more methylene bridges (cross-links) are formed creating increases in the molecular weight of a peptide by multiples of 12 Daltons (Toews, 2008). Chemical reactions that occur with cross-linked peptides can result in incomplete fragments upon collision-induced dissociation, requiring additional targeted experiments to correctly identify those peptide fragments (Sutherland, 2008). This necessitates the creation of specialized software that takes into consideration these mass effect changes before correct peptide identification can be made, a daunting task considering the multitude of combinations possible due to fixation (Leitner, 2010). The presence of cross links may also result in intra-protein peptide combinations as well as portions of peptides between different proteins, making an already difficult task even more complicated. Research into the mass spectrometry analysis of formalin-fixed, paraffin-embedded tissue has shown that comparable data with matched fresh frozen material can be done using principles learned from antigen retrieval and immunohistochemistry. The original findings that compared the numbers of proteins identified by mass spectrometry analysis between matched formalin fixed, paraffin embedded and frozen tissue sections showed the former to consistently be quantitatively less than the latter (Crockett, 2005, Bagnato, 2007 and Guo, 2007). Despite claims touting the feasibility of mass spectrometric analysis on formalin-fixed paraffin-embedded tissue, complete concordance of protein inventories with matched frozen tissue has yet to be achieved, leading to speculation that material not detected from formalin-fixed, paraffin-embedded tissue may be due to incomplete lysis of cross links and either incomplete or biased protein extraction (Nirmalan, 2008). Research on fixed biospecimen material continues with the goal that either a mechanism of improved recovery will be found, a more molecular friendly method of fixation developed, or an alternative means of diagnostics created that is compatible with both surgical pathology and molecular assays.

4.1 Barriers to the molecular profiling of clinical specimens - preanalytical variables

Complicating the implementation of molecular analysis on fixed tissue biospecimens is the growing awareness that a number of preanalytical factors may unduly influence the characteristics of certain molecules prior to fixation. Since the intent of assaying a tissue biospecimen is the characterization of molecules reflective of a cancer cells' *in vivo* state, it stands that any factor that alters that state is undesirable. Unfortunately, a number of factors have now been recognized that may introduce unintended molecular variation to the biospecimen and include the type of surgical procedure, warm and cold ischemia, time to fixation, tissue thickness and rate of fixative penetration. These variables predominantly

affect the most labile molecules, namely mRNA and protein phosphorylation status (Sprussel, 2004 and Espina, 2008) and occur early in the procedure (Miyatake, 2004 and Schlomm, 2008). The first of these variables, the type of surgical procedure, can influence a cell's molecular signature due to the initiation of hypoxia. As the specimen is being excised, vessels are sequentially ligated before the entire specimen is ready to be extirpated. The type of procedure can influence the molecular signature, as the shorter, quicker procedure will have less of an influence than procedures that take longer, time wise. An approach that is becoming more popular because it is less invasive and therefore results in a shorter hospital stay, is laparoscopic surgery. For resections of colon cancer specimens, the durations of surgery increases 55 minutes from that of an open surgical resection (COSTG, 2004). With the introduction of robotic assisted surgery, a procedure rising in popularity because of benefits like less blood loss for the patient, the duration of ischemia can increase between 30 minutes to 1.5 hours in prostatectomy specimens. These short changes in time may not be significant enough to change the levels of proteins, but can unduly influence the expression profile signatures of the more labile mRNA transcripts (Ricciardelli, 2010). This increase is attributable to a reversal in the sequence of vessel ligation, where in robotic assisted prostatectomy procedures they are done earlier as opposed to later, as in open prostatectomies. Whereas these changes occur prior to the acquisition of the tissue by the department of pathology, other variables influence the molecular signature of cells within tissue post-acquisition. Cold ischemia, or the time the tissue is outside of the body and either frozen or fixed, is the major factor in determining the adequacy of the tissue for further analysis. Although not incorporated into many protocols, the time from receipt to freezing has a strong negative impact on the molecular profile of the tissue specimen (De Cecco, 2009). For tissue that undergoes fixation, the cold ischemia time is longer. For these specimens, once they are received within the department of pathology, they are immersed in 10% neutral buffered formalin. The cells within the tissue however, will remain viable for a limited time, most likely until they become fixed. The cells are now enduring a loss of their blood supply, accumulation of lactic acid with the subsequent decrease in their cellular pH, and changes in temperature (room temperature vs. *in vivo* body temperature), all resulting in biologic stress. In response to this biologic stress, they will react, and in the process their molecular signature will be altered to some degree to this stress. The obvious conundrum is to determine what of the molecular signature can be considered artifactual, i.e., as a complication of this artificially induced stress, and what can be characteristic of the neoplastic state. An added layer complicating these changes is the fact that tissue thickness can lead to regional differences in the specimen's molecular profile. Formalin infuses into tissue at 1 mm an hour. If specimens are not sectioned before they are placed in a container of formalin, the tissue at the center of the specimen will be fixed last relative to the exterior of the specimen. Since the cells in the tissue are still viable until fixation, the cells within the center of the specimen will be responding to their new environment (Stan, 2006). Depending on their distance from the fixative, these cells will experience progressively hypoxic, acidic and nutrient depleted conditions over time (Espina, 2008 and van Maldegem, 2008). As viable cells now under biologic stress, their molecular profile may alter leading to falsely elevated or decreased levels of a putative biomarker relative to those cells in direct initial contact with formaldehyde. The basic tenet when working with clinical specimens is to realize that excised tissue is viable and not only vulnerable but reactive to *ex vivo* stressors, and that an understanding as to their location within the specimen with respect to exposure to fixative should be considered in the data analysis (Espina, 2008).

These preanalytical factors have the potential to lead to significant variability in the molecular signature of cells within a tissue specimen. Although there exists numerous tissue biorepositories within the United States, a major problem with each is the wide variation in tissue collection, processing and storage of these samples and an absence of standardized procedures for each step (National Biospecimen Network Blueprint, 2003). The development of a sample preanalytical code proposed by the International Society of Biospecimen and Environmental Repositories represents a good start towards standardization (Betsou, 2010). Through the compilation of data from tissue biospecimens, and correlation with this proposed grading system of specimen integrity, the factors that play into macromolecule integrity can be identified. Each organ may exhibit differences in the stability of the molecules within their cells, with those possessing digestive type enzymes (e.g., the pancreas) more labile than those without (e.g. skeletal muscle). A previous study indicated that the biopsy, based on the smaller tissue size and exposure to the shorter periods of warm or cold ischemia, to be the optimal tissue biospecimen for molecular analysis. The small size of the biopsy allows for even exposure to fixative, while the actual procedure of acquiring small pieces of tissue are not encumbered by extensive periods of surgically induced ischemia required to excise a diseased organ nor intraoperative procedures that are deemed clinically imperative to the needs of biospecimen collection (Schlomm, 2008 and Espina, 2008).

4.2 Barriers to the clinical profiling of clinical specimens - tissue heterogeneity

Another barrier to molecular profiling of clinical tissue biospecimens is tissue heterogeneity. Human tissue specimens are increasingly being used as the primary source of investigational material for cancer related studies. They offer advantages over cells lines because they are more representative of the diagnosed condition and reflective of the *in vivo* condition, not having undergone numerous passages and the resultant phenotypic and genetic drift. Properly collected and annotated, they can avert the problems of misidentification, a relatively widespread situation wherein certain cell lines actually correspond to other cell types than what they are designated to be (Buehring, 2004)). Despite extensive work on cell lines, the realization that the cell of origin may actually be a contaminant would be disastrous for any investigator. However, using tissue itself is fraught with problems. The integrated architecture of tissue means that the targeted cell of interest will vary with respect to the overall cell volume, even in normal tissue (Figure 1). In tumor samples, the same problem exists (Enkemann, 2010). Depending on the type of tumor, the percentage of non-tumor cells can also be less than 50%. Although in grossly solid areas of tumor tissue colon cancer, lung cancer and breast cancer can compromise over 80% of the tumor, prostate cancer is notable for interdigitating between normal glands, possibly biasing any subsequent findings based on such a tissue biospecimen (Figure 2). The presence of contaminating normal cells in a tumor sample can have the affect of dampening a signal or mask the detection of a potential biomarker molecule. The presence of segments of nucleic acids originating from contaminating normal cells can have the untoward affect of decreasing the amplitude of a deleted gene in a tumor sample (Mojica, 2007). Ideally, any work done on a sample should ensure that it is a pure, or close to pure cell population of the desired cell type, so that retrospective analysis trying to deconvolute the data does not have to be performed (Tureci, 2003 and Shen-Orr, 2010). The development of the laser capture micro-dissection tool has provided an answer to tissue heterogeneity. With this machine or one of its congeners, specific cell types can be visualized, identified and then procured,
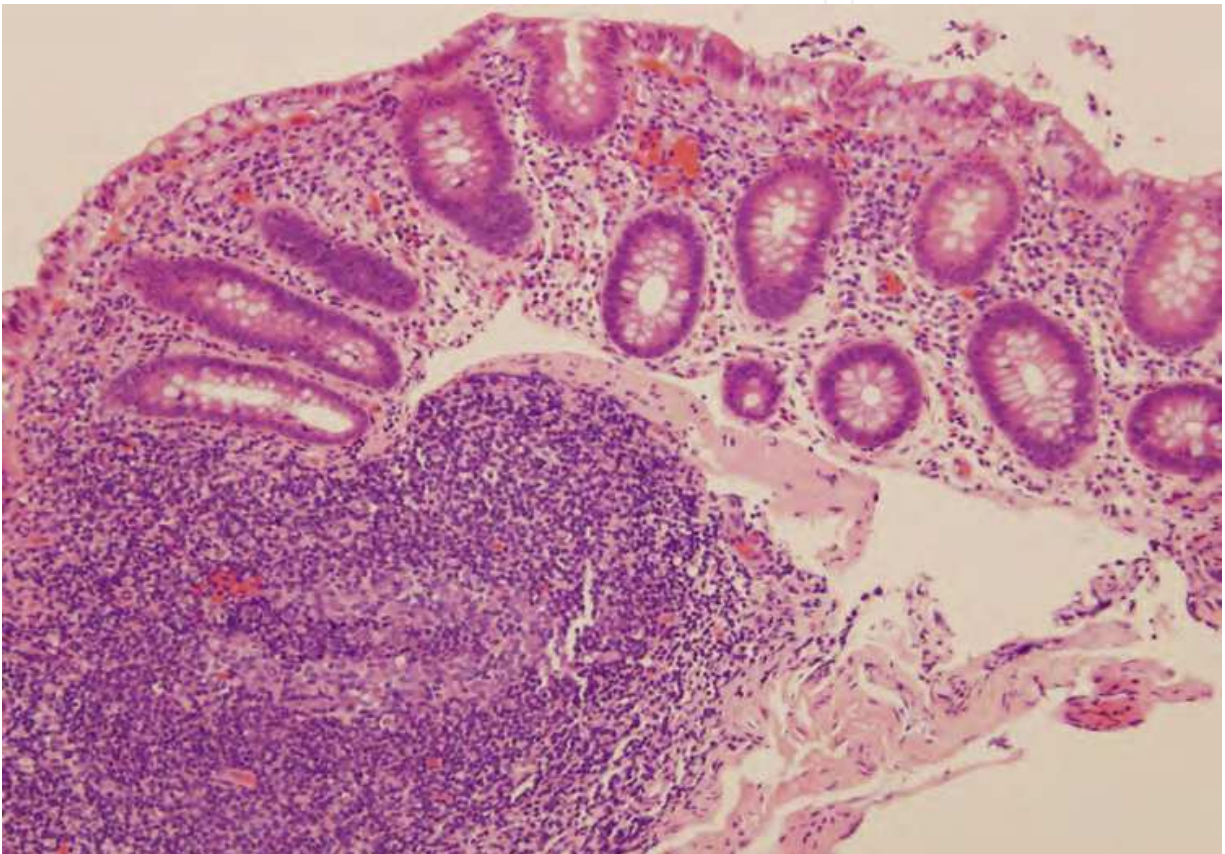
Fig. 1. Histology of normal colonic mucosa. The glands and surface consist of epithelial cells, while the lamina propria contains chronic inflammatory cells. Interspersed in the mucosa are lymphoid aggregates (left side of figure), which cannot be readily discerned at the gross examination level. Without microscopic examination, the proportion of targeted cells, in this case colonic epithelial cells, could account for less than 50% of the cells in sample. Hematoxylin and Eosin stain, 10X
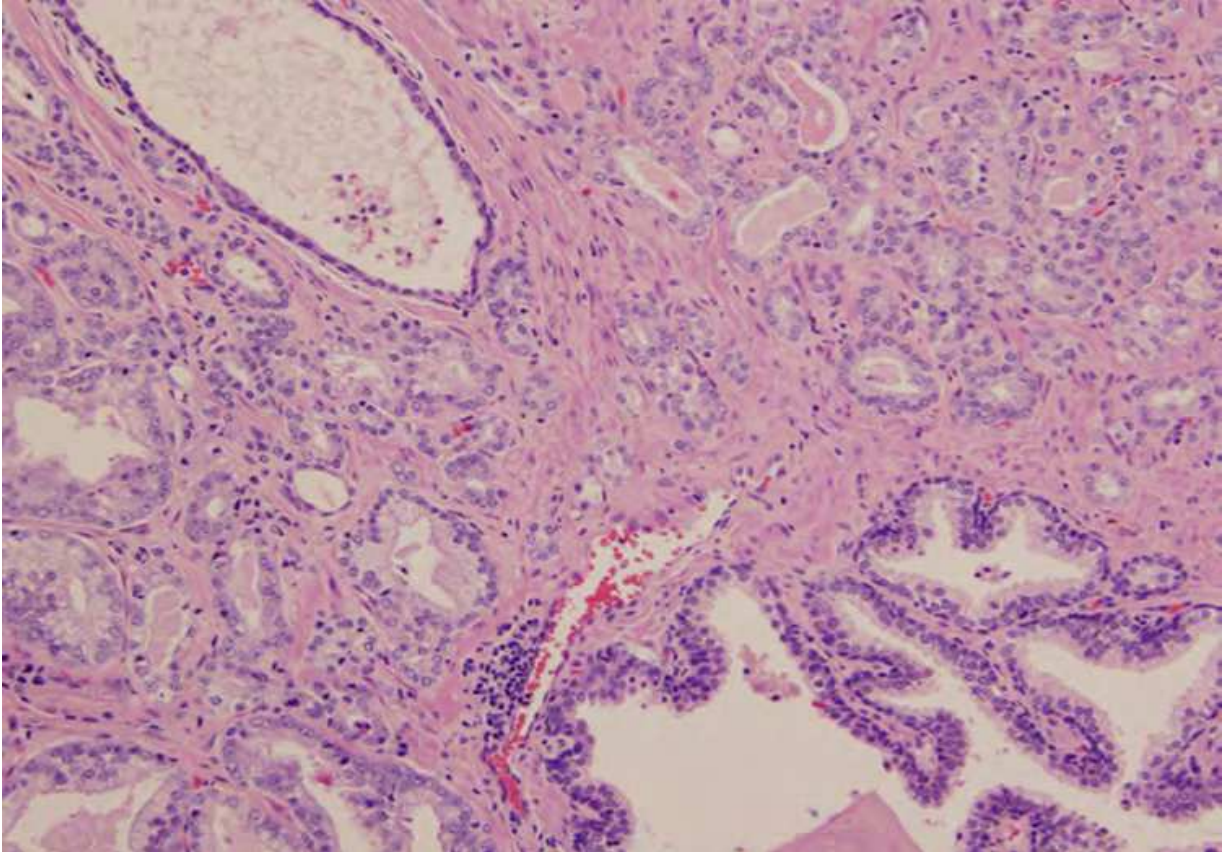
Fig. 2. Histology of prostate cancer. Tumor is in the upper right hand corner, while normal glandular prostatic cells and stroma make up the remainder of the specimen. Hematoxylin and Eosin stain, 10X.

leaving the unwanted contaminating cells with the tissue. It works for both frozen tissue specimens and formalin fixed paraffin embedded tissue. A major drawback with its use however, is the time consuming nature of manually procuring the wanted cells. This issue has been addressed with the development of automated programs, that coupled with cell recognition software, have alleviated the overall time needed to select cells from tissue specimens. The other major drawback with this instrument has been its high start-up cost. Most machines have a six figure price tag, but newer, cheaper versions have been developed. An alternative method has been the use of immunomagnetic beads for the recovery of targeted cells (Mojica, 2006).This approach starts with fresh tissue specimens, that is, before they are fixed, and after a series of manipulations, recovers a highly enriched collection of cells. This method is cost effective, and does not involve any significant expenditure. It has advantages over using straight (un-enriched) biopsy samples in that it can enrich for a targeted cell population. Simple adjustments to the procedure can
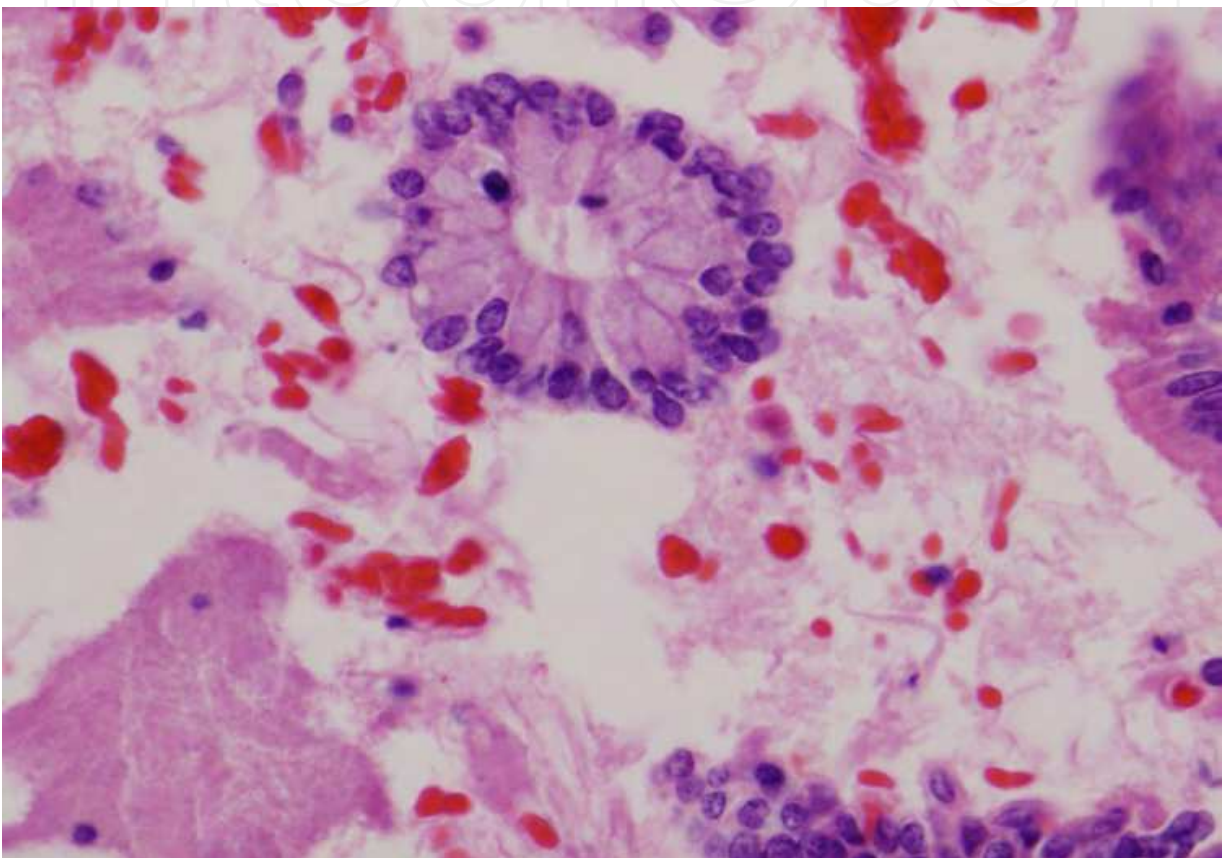
Fig. 3. Cells exfoliated from colonic tissue with no enrichment. Note presence of red blood cells and mucus. Hematoxylin and Eosin stain , 40X.
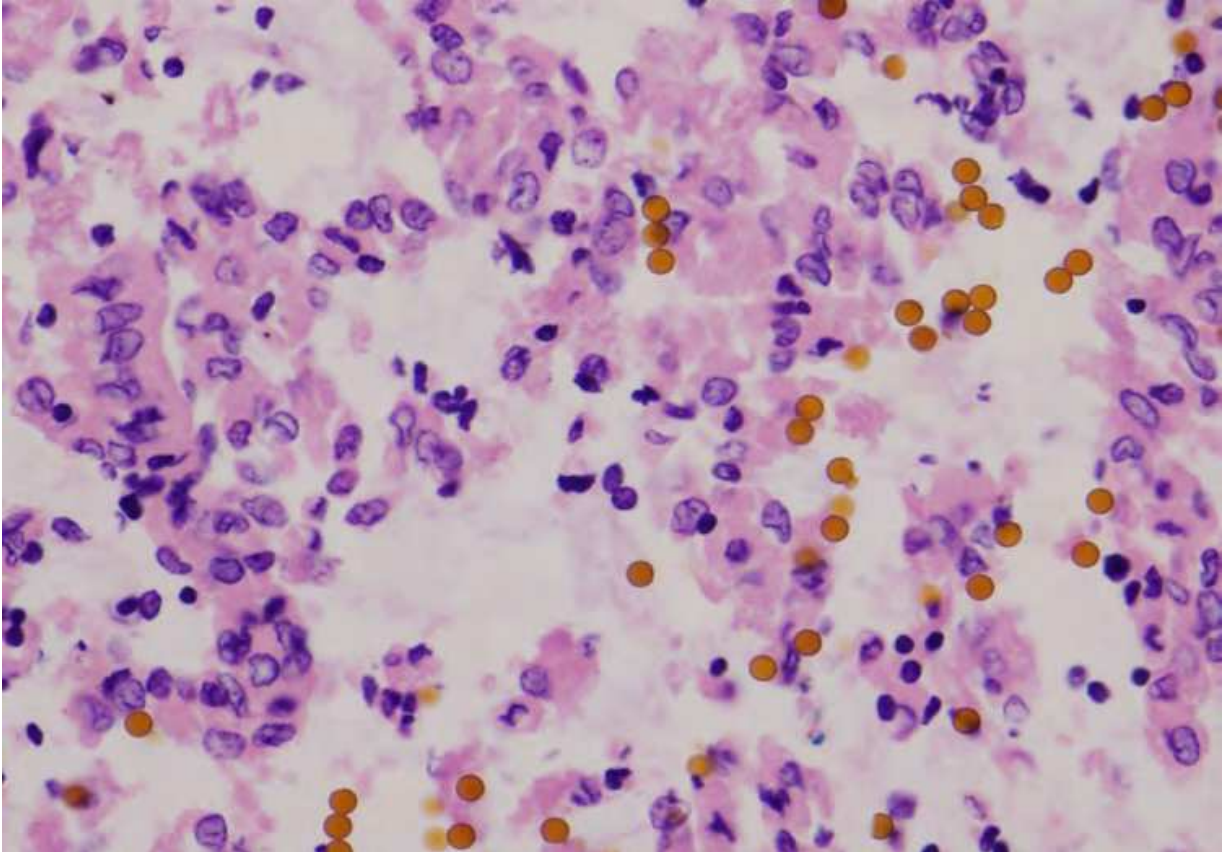
Fig. 4. Recovered cells from enrichment described in text. Yellow dots are immunomagnetic beads. Hematoxylin and Eosin stain 40X

targeted cell population. Simple adjustments to the procedure can be made to eliminate the presence of red blood cells, whose presence is significant in tissue biospecimens (Figure 3). This is particularly important for proteomic, and not so much for nucleic acid studies, as red blood cells have a number of proteins within their cytosol that may contribute to complicate any subsequent un-enriched lysate (Pasini, 2006, and D'Alessandro, 2010). The result of such enrichment is an expansion in the detection of the proteins from the targeted cell (dePetris, 2010). The use of several washes helps eliminate the presence of other contaminating substances, using as an example, excess mucus from colon specimens. Mucus itself is not detrimental to downstream analysis, since it is the secreted product of certain types of colonic epithelial cells. It is however, the location whereby commensal organisms like bacteria habitate, and their inclusion in any sample for downstream analysis could lead to confounding genetic or proteomic results (Qin, 2010). Since the technique can be done on fresh tissue specimens, the problems associated with formalin enumerated earlier are not encountered. Finally, the use of the ber-Ep4 immunomagentic beads are commercially

available and have been for several years. As ber-Ep4 recognizes an epitope in epithelial cells, this method works nicely as a means for their procurement from specimens with the intent to isolate epithelial cells from the underlying tissue (Figure 4). The overall technique however, is not restricted to ber-EP4, as other antibodies with specificities to other cell surface membrane proteins can be conjugated to the magnetic beads. One of the limiting factors however, is the relative paucity of antibodies available to select from that may be used to positively enrich for a specific cell type from a heterogeneous population. This dearth is attributable to the limited knowledge of the proteome of the plasma membrane, which in turn is due to the difficulties associated with examining molecules with hydrophilic and hydrophobic properties. When the plasma membrane proteome is not known for a specific cell type, an alternative but reportedly equally effective approach would be through negative enrichment, where contaminating cells are targeted and removed from the sample population. This has been used for the examination of sputum with respect for lung cancer diagnosis (Qui, 2008). In cytology, the presence of pulmonary macrophages is essential to document that the material has cells from the lung, and not just the oral cavity. These same cells however, would contribute to the heterogeneity of the sample for any subsequent molecular analysis. Their selection, and that of neutrophils using anti-CD-14 and anti-CD-16 immunomagnetic beads can lead to a significant improvement in the numbers of bronchial epithelial cells recovered. An alternative to using antibodies are aptamers. Aptamers are short single stranded nucleic acid oligomers that can take on a variety of three dimensional shapes, thus allowing them to bind to a wide variety of molecules, with binding affinities similar to monoclonal antibodies (Kim, 2009). One advantage of using aptamers over antibodies is their relative stability. Another is the ability to work with cells without knowing specific proteins. This approach would thus be an alternative to the extensive plasma membrane profiling that would be required in order to identify differentially expressed proteins between normal and tumor cell populations.

5. The advent of molecular analysis in cancer tissue specimens

Despite all these roadblocks to using clinically derived tissue specimens for molecular analysis, their use in clinical cancer care already exists. A perfect example is the use of monoclonal antibodies created to antagonistically bind and inhibit the plasma membrane protein Epidermal Growth Factor Receptor (EGFR) in colorectal cancer and non-small cell lung cancer (Plesec, 2009 and Wang, 2010). Although the EGFR receptor can be recognized by immunohistochemistry in tissue specimens, this assay has been found to not be a reliable means of determining those likely to respond to this type of therapy. However, the identification of those patients most likely to respond is imperative in order to avoid any unnecessary side effects, treatment related costs and delays in the administration of a more effective therapy. Since immunohistochemistry was deemed unreliable as a mechanism that could select patients for EGFR therapy, downstream components of the EGFR signaling cascade were examined. The result of these investigations was the finding that mutations in the KRAS gene could identify those patients who would not be responsive to monoclonal antibody therapy directed at the EGFR receptor. In unselected patients, the number of patients that responded to cetuximab, the antibody directed against EGFR, was between 10-20%, but when patients were selected for treatment based on the criteria of no KRAS mutations, this percentage rose to 60%. The detection of these mutations can be accomplished by a number of different methods, the common denominator being that they

evaluated the DNA sequence of the KRAS gene within tumor cells. The issues previously described associated with DNA and formalin fixation now have practical clinical relevance. Again, these issues relate mostly to template degradation and tissue purity. For the former, the amplified product must be designed to be small enough to account for degradation yet long enough to enable specificity. For tissue purity, most laboratories require the tumor component in the tissue to be equal to or greater than 75%. The presence of contaminating normal cells raises the possibility that the DNA from these cells may be assayed instead of the tumor cell's DNA, resulting in a normal sequencing electropherogram or false negative finding for the relevant mutations. In some specimens the amount of contaminating normal cells is significant, and although laser capture microdissection can be used, the transference of this predominantly research tool to the clinical setting is prohibitive due to its labor intensive nature. A promising approach is the technique of "cold-PCR", wherein mutant alleles in tumor cell can be amplified and subsequently sequenced when specific alleles from these tumor cells comprise only a minority population in a tissue sample (Li, 2008). A recent report documented the ability to detect mutations in tumor cells when they constitute only 20% of the tissue sample using the technique of Cold-PCR (Yu, 2011). If no other developments in tissue preparation are introduced, this method may represent an approach that may become a standard assay for tissue biospecimens.

6. Changes in the clinical arena warranting changes in biospecimen preparation

Although the technique of cold-PCR will prove very useful in the evaluation of tumor specimens for specific mutations, its application or modification to whole genome profiling is unknown. Despite an ever expanding database of information that is characterizing oncogenesis, there still remains a significant amount of knowledge to be gained. A recent initiative to correlate the molecular signatures of tumor tissue specimens with the corresponding cancer patient's clinical parameters underscores the need to identify biomarkers that will aid in cancer care. As such, the tissue biospecimen will continue to play a significant role in cancer diagnosis and treatment. One lesson that is becoming increasingly more apparent is that material that is not fixed serves as the best medium from which to start (De Rienzo, 2010 and Jimeno, 2010). This is a reiteration of what has previously been documented, but applied to actual biospecimens. The nature of the unfixed specimen in the form of fine-needle aspirates however, has one major drawback. The technique of fine needle aspiration involves inserting a needle into a mass, and pulling the needle in and out of the mass so that tissue fragments are obtained. Since the tool is a needle, the tissue fragments are minute. Obtaining a diagnosis based on cytology requires extensive training in pathology and its subspecialty, cytopathology. Without the architecture, the presence of malignant cells can be diagnosed, but the diminutive nature of the specimen sometimes precludes assigning what type of tumor it is. This is particularly true for tumors of the lung, where a process similar to fine needle aspiration, in terms of quantities of cells recovered, is performed. Whether the procedure is a bronchoscopy with material obtained as washings or brushings, or a trans-thoracic needle biopsy, the material recovered will be diminutive. Again, the diagnosis of malignancy can be made, but for poorly differentiated tumors, the classification into adenocarcinoma, squamous cell carcinoma or large cell undifferentiated carcinoma, which is now often requested to help guide therapy, will be extremely difficult if next to impossible without a large enough piece

of tissue to showcase architecture (Wallace, 2009). For adenocarcinoma, the presence of five or more vacuoles in two consecutive high power fields is required to differentiate adenocarcinoma from large cell undifferentiated carcinoma. However, the diminutive nature of these specimens may not even fulfill this size criterion. If not enough material is available to make a definitive diagnosis, the patient may require another invasive procedure that may aim to get tissue instead of cells. A similar situation is occurring with the biopsy of small kidney tumors where cryoablation instead of resection will be performed. Although attempts to obtain tissue so that a histologic diagnosis can be made, often times the specimen is too small and not recovered after tissue processing. The absence of an established diagnosis means that should another mass lesion arise in a patient treated by cryoablation, it will not be known if the mass represents a metastatic or primary tumor Although tumors of unknown origin can be worked up through conventional immunohistochemistry, the absence of a previous diagnosis incurs additional costs for this work-up. Thus, at today's medical environment, a histologic diagnosis is still imperative, and thus in order to optimally process any specimens in the near future, tools will need to be developed that are capable of allowing the evaluation of these cells at both the cytologic and molecular level.

In order to circumvent the problem of enabling a diagnosis on small biopsy specimens all the while providing some material for molecular analysis, investigators have begun designing platforms capable of recovering specific subsets of cells from clinical specimens (Weigum, 2010, Wan, 2010, and Sun, 2010). These platforms are ideal for taking on the problems of small amounts of cells or tissues. Although immunohistochemistry is an extremely useful diagnostic tool in the laboratory, the amount of tissue used per assay is significant. When a battery of stains is needed to be performed, each 4 or 5 micron thick section may end up exhausting the amount of recoverable tissue. Precious tissue can also be lost at the microtome step, where some material may be lost just due to the process of aligning the block and blade. Flow cytometry has been used extensively for years for hematologic malignancies, but its requirement for ample amounts of starting material negate its consideration as a tool for biopsy specimens. Thus, platforms based on microfluidics appear to be best suited to interrogate these small biopsy specimens. Using techniques similar to the one previously described above that help enrich for targeted cells, these enriched cell populations can then be introduced to a microfluidic platform for subsequent evaluation. One noticeable limitation of these platforms however, is the absence of the ability to visually evaluate these cells. Although the enrichment approach previously described is capable of selecting for epithelial cells from the heterogeneous cell population that comprises tissue, the epitope recognized by the ber-EP4 antibody can be present in both non-neoplastic and malignant epithelial cells. Thus, until plasma membrane proteins are identified and characterized that are specific for either normal or neoplastic epithelial cells, enrichment is limited to epithelial cells. This is not so much a limitation in certain tumors like colonic adenocarcinomas, where the tumor mass effaces the surrounding tissue so that it is composed of tumor epithelial cells and surrounding stroma, but will not be as effective in other tumors like the prostate, where the tumor cells infiltrate between normal prostatic glands and stroma. For microfluidic platforms, the need to visualize cells will allow investigators whether to attribute molecular findings appropriately to neoplastic cells or not. At the clinical level, the ability to visualize cells allows for a diagnosis, and determination whether the biopsy contains tumor cells and thus is representative of a mass versus no tumor cells and therefore not representative of a mass.

In the development of such a platform, cytologic examination plays the vital role of stratifying whether the cells are tumor or not. Identifying the type of tumor is not so vital as the cells, or specifically their contents, can be interrogated through a variety of downstream assays. The platform needs to be designed such that the cells are not permanently immobilized on the glass slide. Once visualized, they should either be able to be eluted out as intact cells, lysed so that the molecules from them can be assayed, or have a lab-on-a chip assay integrated to a portion of the slide. Thus, such a microfluidic platform should allow for conventional cytologic based examination for screening purposes, followed by molecular interrogation for tumor designation. A period of molecular annotation will take place within the next decade wherein reliable biomarkers will be discovered and validated for specific tumor types. The transition to molecular identification of tumor tissue has already begun and has shown early success in helping determine the site of origin in several instances (Ismael, 2006, Greco, 2010, and Monzon, 2010). Molecular tumor classification is not yet a reality, with histologic examination still the gold standard for diagnosis (Kotsakis, 2010). However, in the next decade, when molecular analysis of tumor specimens will occur concurrent with histologic examination, a gradual shift in emphasis may occur. With Personalized Medicine, the identification of target molecules may eventually become more important for cancer treatment than histology.

6.1 An integrated approach to optimize the biospecimen for cancer care

Taking into consideration all the factors that may bias the macromolecular profiles of a specimen, a tentative approach can be postulated that would recover cells bearing the most similar molecular disposition to their *in vivo* counterparts from clinical material. To address preanalytical bias, the specimen most suited for analysis may be the biopsy. Clinically, the patient undergoing a biopsy of a tumor would not be subjected to the depth of anesthesia that the patient undergoing a surgical resection would be for the same tumor. The degree of preparation of the patient is less, with the process most often an out-patient procedure. Therefore, confounding issues that potentially could influence, but are hard to confirm, the molecular profile of a cell, like administration of intravenous fluids, antibiotics and anxiety to name just a few, are not introduced (Compton, 2006). For issues related to warm ischemia, the short time required to obtain a biopsy is also preferred to the comparatively longer time required to excise a portion of organ with tumor (Schlomm, 2008). Once the biopsy is obtained, the next step would be sample stabilization, an attempt to immediately try to preserve the molecules within the cells. For the more labile molecules like RNA, this can be achieved by immersing the specimen in RNA Later (Mutter, 2004) or for proteins, through heat denaturation (Svensson, 2008). For the latter, the use of heat inactivation helps to preserve post-translational modifications, a form of intercellular signaling with a transient time frame within proteins that can be lost or altered during cold ischemia, or the period after extirpation (Rountree, 2010). Once stabilized, the cells should be disaggregated from the tissue and unwanted elements and contaminating normal cells eliminated through some form of enrichment. It should be noted that at no point in this proposed procedure fixation with formaldehyde is involved. The cells then should be examined cytologically, to confirm that the targeted cells are indeed present, that they are enriched and to what percentage they constitute the sample. This should be done on some fluidic based platform, so that these same cells can be further examined at the molecular level after microscopic diagnostic evaluation. The cells are then eluted and their contents extracted for downstream

molecular analysis. Alternatively, other methods may develop wherein diagnostics can be performed on the immobilized cell. Through the development of the above protocol, the maximal amount of informative data can be extracted from the biospecimen, with a combination of traditional anatomic (cytologic) related diagnostics and future molecular interrogation for treatment guidance obtained from an optimally recovered specimen.

6.2 Biospecimens as the focal point of cancer care

The development of a platform capable of enriching unfixed, preserved tumor cells from tissue specimens will enhance clinical research. For some years now it has been known that one of the major obstacles to progression in the post-genomic era has been the absence of high quality biospecimens. An attempt in the previous decade to obtain 500 cases each of specific types of brain, lung and ovarian cancer for gene sequencing initially failed at the accrual stage. Although archives in pathology departments have numerous amounts of preserved tissue specimens, issues with percentage of tumor content, necrosis or even proper consent have become factors preventing their use at a national level. Differences in the procurement, handling and storage of samples may also play a role in the differences noted between similar tumors from different institutions. Nevertheless, the National Cancer Institute has embarked on an initiative to address those issues related to poor biospecimen procurement and to rectify them. The need for high quality biospecimens is reflected in their incorporation in innovative study designs for clinical trials, like the new phase 0 trial. In this new type of study, the need for patient tumor tissue is necessary in order to determine the biological effectiveness of the therapeutic agent being tested. In phase 1 trials the need for high quality biospecimens is also warranted, to match the administration of specific agents to patients possessing a specific molecular signature, deemed through previous experimentation, to be the target of those agent(s). This approach identifies useful therapeutic agents in a subset cohort from the general cancer population, where if an empiric approach had been used, the agent may not have resulted in a significant enough number of patients to warrant further testing. Molecular testing should also be able to identify patients who will be resistant to certain agents, thus guiding the clinician to alternative approaches and avoiding any unwarranted side effects and unnecessary costs. In the future, the tissue biospecimen will serve many roles, with two being the basis of future molecular, proteomic and/or metabolomic research and the other being the starting point for the treatment of the cancer patient. The development of newer biotechnological tools will certainly result in a continuing evolution of the tissue biospecimen's role in the treatment of cancer.

7. Conclusions

The role of tissue biospecimens in cancer care has evolved over time from observation and little impact, to anatomic and molecular diagnostics with high impact on guidance of clinical treatment. This evolution has come about based on the changes in the evaluation of the specimen, from humble beginnings wherein only the gross anatomic features were noted in malignant tissue and the patient's clinical course observed, to the biotechnological revolution and the high throughput capabilities created that can profile molecular alterations within cells, identify therapeutic targets and selectively lead to the administration of reagents best suited for each individual cancer patient. Advances in

science continue to influence the evaluation of the biospecimen, with an urgent need to develop and adapt newer approaches capable of maximizing the amount of informative data that can be derived from these samples. The importance of the tissue biospecimen in the care of the cancer patient has grown dramatically. It is safe to assume its role in the care of the cancer patient of the near future will become even more vital than it is today.

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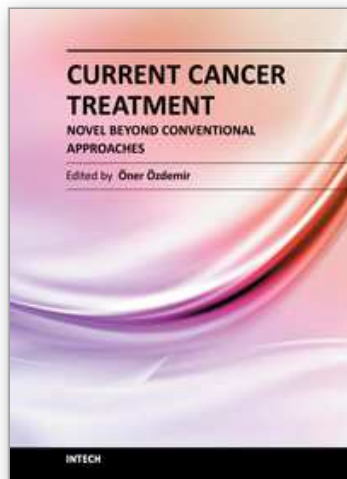
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Currently there have been many armamentaria to be used in cancer treatment. This indeed indicates that the final treatment has not yet been found. It seems this will take a long period of time to achieve. Thus, cancer treatment in general still seems to need new and more effective approaches. The book "Current Cancer Treatment - Novel Beyond Conventional Approaches", consisting of 33 chapters, will help get us physicians as well as patients enlightened with new research and developments in this area. This book is a valuable contribution to this area mentioning various modalities in cancer treatment such as some rare classic treatment approaches: treatment of metastatic liver disease of colorectal origin, radiation treatment of skull and spine chordoma, changing the face of adjuvant therapy for early breast cancer; new therapeutic approaches of old techniques: laser-driven radiation therapy, laser photo-chemotherapy, new approaches targeting androgen receptor and many more emerging techniques.

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