DETECTION OF BLADDER TUMOR RECURRENCE BY FOURIER TRANSFORM INFRARED SPECTROSCOPY AS A NOVEL METHOD

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

DETECTION OF BLADDER TUMOR RECURRENCE BY FOURIER TRANSFORM INFRARED SPECTROSCOPY AS A NOVEL METHOD

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Bladder cancer is one of the most common urogenital cancers worldwide. Two techniques commonly used for bladder cancer diagnosis are urine cytology and cystoscopy. Cytology is not sensitive for detecting tumors. Cystoscopy is an invasive technique which disturbs patient comfort. In the current study, we used Fourier transform infrared (FT-IR) spectroscopy as a novel method which is rapid and non-invasive to investigate the bladder tumor recurrence using the bladder wash samples collected in the course of control cystoscopy. This study is unique since it is the first one to use the bladder wash sample in the diagnosis of the bladder tumor by using FT-IR spectroscopy.

Molecular investigation of the FT-IR spectra revealed many differences between control and tumor samples such as a considerable increase in protein, carbohydrate and nucleic acids content, and changes in protein and carbohydrate structure. On the basis of the spectral differences, cluster analysis was performed to differentiate between the control and tumorous spectra and we reached to an overall sensitivity (including all individuals with tumor) of 91.8%, a PUNLMP sensitivity of 83.3% and a papilloma sensitivity of 77.8% in spectral range 1444-1457 cm^{-1}. Other spectral ranges also gave similar results. Our results showed that FT-IR spectroscopy can be used to detect the bladder tumors in bladder wash sample with higher sensitivity compared to cytology.

In summary, we propose the utilization of the FT-IR spectroscopy for the detection of bladder tumors since specific spectral regions might be used as effective markers for the diagnosis.

Keywords: FT-IR, bladder tumor, bladder wash, cluster

ÖZ

YENİ BİR METOD OLARAK FOURIER TRANSFORM INFRARED SPEKTROSKOPİ KULLANILARAK MESANE TÜMÖR REKÜRENSİNİN SAPTANMASI

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Mesane kanseri dünyada en sık görülen ürogenital kanserlerden birisidir. Mesane kanseri tanısında sıklıkla kullanılan iki teknik idrar sitolojisi ve sistoskopidir. Ancak sitoloji tümör teşhisinde yeterince duyarlı değildir. Sistoskopi ise hasta konforunu bozan girişimsel bir yöntemdir. Bu çalışmada kontrol sistoskopi sırasında alınan idrar yıkama örneklerinden mesane tümör rekürensinin saptanması için yeni bir metod olarak, hızlı ve vücut içine girme gerektirmeyen bir yöntem olan Fourier transform infrared (FT-IR) spektroskopi kullanılmıştır. Bu çalışma, FT-IR spektroskopisi kullanilarak mesane yıkama sıvısından, mesane tümör teşhisi yapılan dünyada ilk çalışmadır.

FT-IR spektrumlarının moleküler incelemesi, kontrol ve tümörlü örnekler arasında, protein, karbonhidrat ve nükleik asit içeriğinde dikkate değer artış, protein ve karbonhidrat yapısında farklılaşma gibi birçok değişiklik ortaya koymuştur. Bu spektral farklılıklara dayanarak, kontrol ve tümörlü örnek spektrumlarını ayırma amaçlı olarak cluster analizi yapılmış ve uygulanan yöntemin duyarlılığı 1444-1457 cm^{-1} spektral aralığında tüm hastalarda %91.8'e, PUNLMP hastalarında %83.3'e, papilloma hastalarında ise %77.8'e ulaşmıştır. Denenen diğer spektral aralıklar da bu değerlere yakın sonuçlar vermiştir. Çalışma sonuçları FT-IR'ın sitolojiye oranla daha yüksek bir duyarlılık ile mesane tümörlerinin teşhisinde kullanılabileceğini göstermiştir.

Sonuç olarak, belirli spektral aralıkların cluster analizinde oldukça başarılı tümör göstergeleri olabilecegi ortaya konulduğundan, mesane tümörü teşhisinde FT-IR spektroskopisinin uygulanmasını önermekteyiz.

Anahtar Kelimeler**:** FT-IR, mesane tümörü, idrar yıkama, cluster

To my family,

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1.1.1 General Information About Bladder

The urinary bladder is a solid, muscular, and elastic organ that sits on the pelvic floor in mammals. It is the organ that collects urine excreted by the kidneys prior to disposal by urination. Urine enters the bladder via the ureters and exits via the urethra (Figure 1).

The shape and behaviour of bladder changes depending on its fullness. In males, the base of the bladder lies between the rectum and the pubic symphysis. It is superior to the prostate, and separated from the rectum by the rectovesical excavation.In females, the bladder sits inferior to the uterus and anterior to the vagina. It is separated from the uterus by the vesicouterine excavation. In infants and young children, the urinary bladder is in the abdomen even when empty.

Normal bladder epithelium consists of 4-8 layers of transitional cells. The bladder muscle layer is called detrusor and contains irregular muscle fibers all around the bladder. The outermost part of the bladder is serosa which is covered by the adipose tissue. Serosa consists of collagen and elastic fibers. Inside the serosa lies veins, nerves and ganglions (Baykara, 1999).

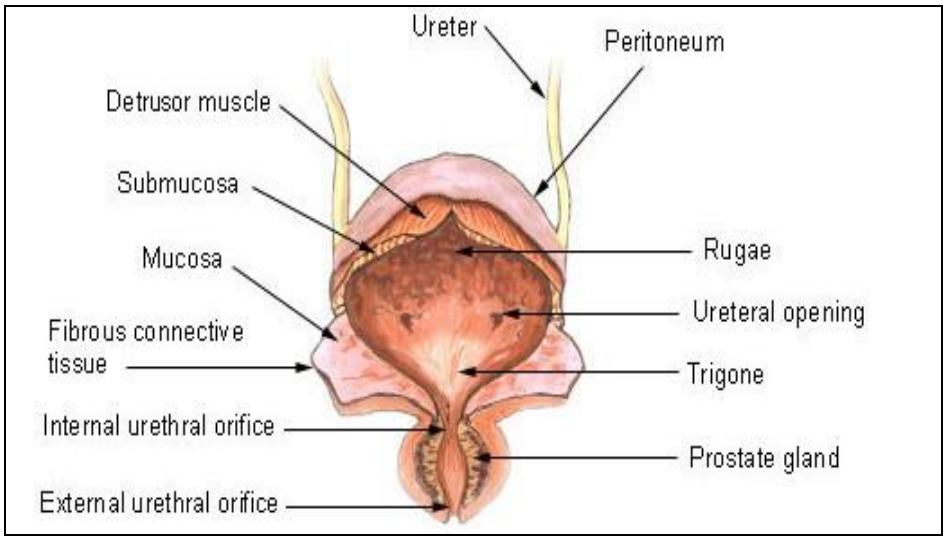


Figure 1: The bladder (http://training.seer.cancer.gov/module_anatomy/images/illu_bladder.jpg)

1.1.2 Bladder Cancer and Its Incidence

Bladder cancer is one of the most common malignancies in human and the most common malignancy in the urinary track. An average of 260,000 new cases of urinary bladder cancer are diagnosed worldwide every year, with an estimated 63,210 new cases in 2005 in the USA alone, with 13,180 deaths (American Cancer Society, 2005). In Europe and the United States, bladder cancer accounts for 5% to 10% of all malignancies among men (Figure 2).

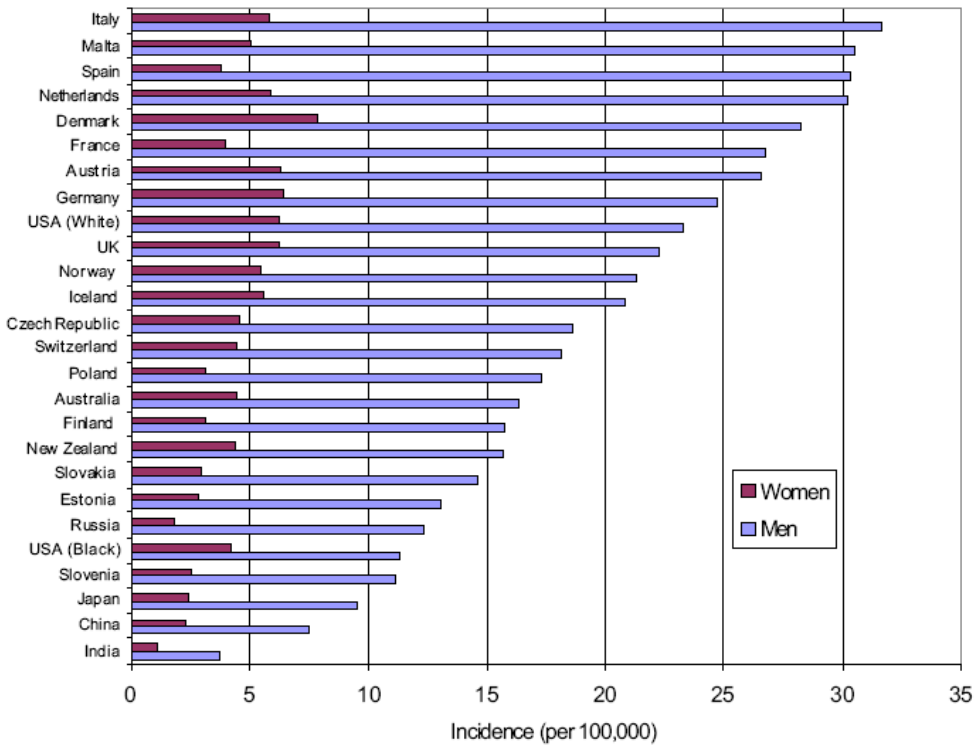


Figure 2: Age-standardized (world) incidence rates of bladder cancer. (Parkin *et al.*, 2002).

In Turkey, according to KIDEM's (İzmir Cancer Registry) database, bladder cancer is the second most common cancer after lung cancer and the incidence is approximately 13/100,000 in men in İzmir (Zorlu *et al.*, 2004). On the other hand, the incidence in women is 1.2/100,000 in İzmir.

Bladder cancer is 3 to 4 times more common in men than in women. On the other hand, it has been suggested that the stage-adjusted survival of bladder cancer in women is worse than men (Mungan *et al.*, 2000). The excess of bladder cancer in men versus women is not fully explained by differences in smoking habits and occupation (the 2 most well-known risk factors for bladder cancer). Surveys of cancer incidence and mortality suggest that parous women have a lower risk of bladder cancer than nulliparous women, probably because of hormonal changes related to pregnancy, and that the risk may decrease with increasing parity (Cantor *et al.* 1992).

Although it is also observed in young individuals, bladder cancer is a disease that is most commonly seen in elderly people. The median age at diagnosis is 65 to 70 years. Fast industrialization lowers the average age. However, the tumors which are seen in younger individuals have better histological features and prognosis. In literature, 1 case which is younger than 10 years, 100 cases which are younger than 30 years and 13 pregnant women are reported (Catalona *et al*., 1992).

1.1.3 Risk Factors of Bladder Cancer

There are various suspected risk factors for the bladder tumor development and progression. The suspected risks differ according to the type of bladder tumor. The superficial tumors which progress slowly is thought to be associated with some genetic predispositions while aggressive transitional cell carcinoma and squamous cell carcinoma are thought to be associated mostly with carcinogenic chemicals.

1.1.3.1 Environmental Factors

The most well-established risk factor is cigarette smoking (Pagano *et al.*, 1995). Occupation is the second most important risk factor and it has been estimated that occupational exposures may account for as much as 20% of all bladder cancer (Vineis *et al.*, 1991). Workers and former workers in the textile dye, rubber and chemical industries have an increased risk for bladder tumor as a result of the exposure to aromatic amines (arylamines), such as benzidine, 2-naphthylamine, 4-ABP, 4,4=-methylenedianiline, 4,4=-methylene-bis(2-chloroaniline), o-toluidine, 4,4=-methylene bis(2-methylaniline), and 4-chloro-o-toluidine (Popp *et al.*, 1992; Schulte *et al.*, 1997). Because of strict regulations, these specific chemicals are now banned from the workplace and contribute minimally to the current incidence of bladder cancer in Western countries. The risk of bladder cancer among workers in such industries should therefore be monitored continuously. If specific plants are suspected, the causative agents

should be identified immediately, preventive measures should be taken, and exposed workers should be screened for bladder cancer for ≥2 decades.

Excess risks have been observed frequently among painters. This is thought to be a result of exposure to possible carcinogenic constituents of paints, such as benzidine, polychlorinated biphenyls, formaldehyde, and asbestos and solvents, such as benzene, dioxane, and methylene chloride (Steenland *et al*., 1999). A moderately increased risk is also found among leather workers and shoemakers. Although the responsible agent is still unknown, exposure to leather dust, dyes, and solvents may play an important role. An excess risk of bladder cancer is also observed in aluminum, iron, and steelworkers, which may be the result of exposure to aromatic amines and polycyclic aromatic hydrocarbons in coal-tar pitch volatiles.

Chronic urinary tract infection (UTI) is associated with the development of bladder cancer, especially invasive squamous cell carcinoma (Kantor *et al.*, 1984). This type of cancer may occur in patients with spinal cord injury in whom chronic cystitis is inevitable. This may be the result of formation of nitrites and nitrosamines by bacterial flora and/or the inflammatory process, which leads to an increased cell proliferation, providing more opportunities for spontaneous genetic mistakes.

Schistosoma haematobium infection has been known to be one of the causes of squamous cell carcinoma of the urinary bladder. The epidemiologic association is based both on case-control studies and on the close correlation of bladder cancer incidence with the prevalence of *S.haematobium* infection within different geographic areas (Bedwani *et al.*, 1998; Gelfand *et al.*, 1967; Lucas *et al.*, 1982; Mustacchi *et al.*, 1958).

Radiotherapy is another risk factor for bladder cancer. In a case-control study of tumors of the bladder in women who had previously been treated for ovarian cancer conducted by Kaldor *et al.* (1995), the risk of bladder tumor was increased for patients who had been treated with radiotherapy or chemotherapy

(thiotepa and melphalan) compared with patients treated with surgery. Moreover, the risk seemed to be much higher in patients who received both.

Other than these, there are many environmental factors which are thought to be associated with the bladder tumor such as the use of analgesics and sweeteners, bacterial, fungal and viral infections, bladder stones and coffee.

1.1.3.2 Genetic Factors

Tumor growth is multifactorial. Under the influence of these factors, malignant change (transformation) develops mainly in two levels which are called initiation and promotion. Initiation is the beginning of carcinogenesis due to the chemicals by causing a lesion on the transitional epithelial cell genome. Many chromosomal abnormalities are associated with initiation. Monosomy 9, trisomy 7, and rearrangements of chromosomes 3, 8, 10, 13, and 17 were reported to have role in the bladder tumor in literature (Fadl-Elmula *et al.*, 1998). Initiation causes irreversible damages in DNA. If the DNA repair efficiency is not enough or the cell replication is accelerated, carcinogenesis starts.

Promotion is the phenotypic appearance of the bladder tumor when the cells pass through initiation and accelerates the proliferation. This mechanism is used to explain how the carcinogens lead to the bladder tumors. Promotion is caused by the continuous and long term exposure to the carcinogen and it is thought to be reversible until the tumor appearance (Gaston *et al.*, 2004). Another possible mechanism in cancer is the inactivation of the tumor suppressor genes. These genes regulate the cell growth and differentiation, prevent the uncontrolled proliferation which is specific to the tumor. One very well known suppressor gene is *TP53* gene which is on chromosome 17p. Another mechanism is deregulation of growth factor related pathways. Overexpression of these genes is also thought to be associated with cell proliferation and tumor development.

1.1.4 Pathology of Bladder Cancer

Normal bladder epithelium consists of 3-7 layers of transitional cells. A proliferation in these layers without abnormality in nucleus or in structure is called epithelial hyperplasy. Metaplasy, on the other hand, is the transformation towards squamous and glandular structures. Squamous metaplasy generally appear in women who are under hormonal therapy and use urethelial catheter for long term. However it is completely benign. In addition, cystitis cystica and cystitis follicularis which have benign properties, are the responses of the bladder to bacterial infection, obstruction and urinary calculus (stone). Glandular metaplasy which shows tranformation towards cancer is the initiator of adenocarcinoma **(**Jost *et al.*, 1993; Heyns *et al.*, 1991).

In epithelial dysplasy, the cell becomes larger, more round and loses its polarity without the proliferation in the cell layers. Dysplasy is subdivided as mild, intermediate and severe. Dysplasies are premalign lesions and as the severity of the dysplasy increases, the potential for the malignancy also increases.

Inverted papilloma is a benign proliferative lesion that is appeared because of the chronic inflamation and an obstruction on the bladder.

Leucoplasy is a metaplasy that is characterised by prominent ceratinisation, acanthosis, cellular atypia and dysplasy. It has been thought that it is formed as a uretelial response to the deleterious stimulus and it is generally accepted as a premalign lesion which can progress to the squamous cell carcinoma in 20% of patients.

CIS of the urinary bladder is defined as a flat, and high-grade cancerous lesion confined to the superficial lining of the bladder. In more than 25% of the patients, CIS coexists with high grade tumors. In 40-83% of patients, CIS progress to muscle invasive carcinoma. Since intercellular connections are loose, the cytology results of 80-90% of the patients are positive (Messing *et al.*, 2002).

1.1.4.1 Transitional Cell Carcinoma (Urothelial Carcinoma)

While 90% of all bladder tumors are transitional cell carcinoma, 70-85% of these are superficial and 10-20% of these show progression and become muscle invasive (Messing *et al.*, 2002). The so-called transitional cells are normal cells that form the innermost lining of the bladder wall. In transitional cell carcinoma, these normal lining cells undergo changes that lead to the uncontrolled cell growth characteristic of cancer.

Transitional epithelium cells have the capacity to stretch and flatten so the calyx and bladder can expand. In the bladder transitional epithelium the cells nearest the apex are rounded. This is a diagnostic feature of this epithelium.

In transitional cell carcinoma, these normal lining cells undergo changes that lead to the uncontrolled cell growth characteristic of cancer. In transitional cell carcinoma, tumor growth can be papillar, solid, infiltrative, nodular and mixed type. The most common type of bladder carcinoma is papillar.

1.1.4.2 Squamous Cell Carcinoma

These cancers originate from the thin, flat cells that typically form as a result of bladder inflammation or irritation that has taken place for many months or years. The prevelance in the world varies. In England, it accounts for 1% of all bladder tumors, in USA 3-7%, while in Egypt accounts for 75% of all bladder tumors. The reason for this is the *Schistosoma haematobium* infection. The median age of the tumors because of this type of infection is 10-20 years earlier than transitional cell carcinomas. In addition, they have more benign prognosis and the risk for the metastasis of the lymph node and distant points is lower. Other than the infection, squamous cell carcinomas develop because of chronic irritations such as the long term catheter usage and kidney stones. The prognosis for this type of squamous cell carcinomas is generally worse (Messing *et al.*, 2002).

1.1.4.3 Adenocarcinoma

These cancers develop from cells that make up glands. Glands are specialized structures that produce and release fluids such as mucus. Adenocarcinoma is a cancer originating in glandular tissue. In the United States, urothelial carcinomas account for more than 90% of all bladder cancers. Squamous cell carcinomas make up 3-8%, and adenocarcinomas make up 1-2% (Messing *et al.*, 2002).

There are also some cancer types in which different cancer types coexist. The most common one is the coexistence of transitional cell and squamous cell carcinoma. Bladder epithelial tumors which are rarely seen, are villous adenomas, carcinosarkomas and melanomas. Non-epithelial tumors, on the other hand, are pheochromositomas, lymphomas, choriocarcinomas and some mesenchemial tumors (Grossfeld *et al.*, 2004).

1.1.5 Biological Behaviour of The Bladder Tumor

The bladder tumors spread is mostly by direct invasion. The tumor in bladder mucosa first spread to basal membrane then to lamina propria and then to muscle tissue. After muscle tissue, it can spread outside the bladder. Since the tumors which invade the lamina propria meet lymph vessels and veins, they can metastase to regional lymph nodes and distant organs.

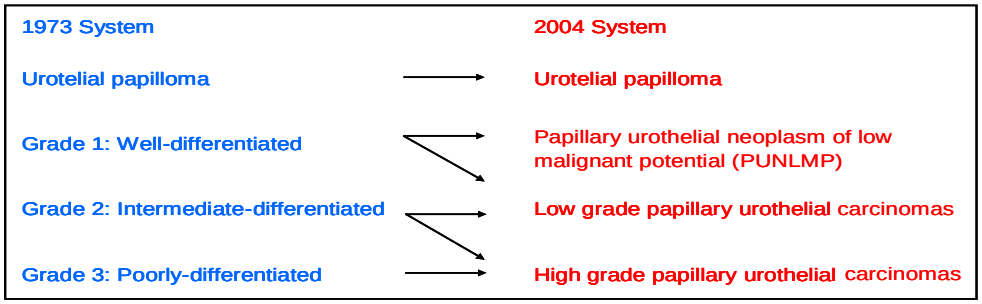
55-60% of all bladder tumors which are diagnosed for the first time, are well-differentiated or intermediate-differentiated superficial and papillar transitional cell carcinomas in USA (Messing *et al.*, 1995). After endoscopic resection, recurrence happens in most of the patients and 16-25% of these recurrences are higher grade tumors (Proud *et al.*, 1992). 40-45% of the bladder tumors which are diagnosed for the first time, are high grade tumors and half of these are already invaded muscle or worse.

1.1.6 Histological Grading of the Bladder Tumors

There are two classification system used for the classification of the bladder tumors: The traditional TNM classification for the tumor staging and the WHO classification system for the tumor grading. These systems relies on pattern recognition and nomenclature for reporting biopsies (Mitra *et al*., 2005). Grading system has taken its final shape in 1998 in WHO/ISUP meeting and published in 2004 (Table 1).

There is strong relationship between grade and stage of the tumor. Grade designation describes a tumor's aggressiveness by stating the differentiation level of the tumor cells. High-grade cancers grow faster and are more likely to invade surrounding tissues. There is a strong relationship between the prognosis of the tumor and the grade, yet not as strong as the relationship between the stage and the prognosis.

Table 1: The WHO 1973 and 2004 classification of the bladder tumor



In the 2004 classification, 4 class are used similar to 1973 classification. However they are stated according to the potencial malignity or carcinoma instead of grade.

Papilloma (grade 0) is the papillary lesion which is surrounded by the normal bladder mucosa which has normal fibrovascular nucleus. There is no increase in

the epithelium cell number or an histologic abnormality. Generally, it does not recur after resection.

Well-differentiated (Grade 1) tumors are classified as papillary urothelial neoplasm of low malignant potential (PUNLMP) or in some cases as low grade papillary urothelial carcinomas in the 2004 classification. These tumors contain fibrovascular core; there is an increase in cell number and they contain very few mitotic activity. They have predisposition to recur and the recurrence has generally higher grade and stage (Cheng *et al*., 1999).

Intermediate-differentiated tumors (grade 2) are classified as low grade papillary urothelial carcinomas or in some cases as high grade papillary carcinomas in the 2004 classification. These tumors contain larger fibrovascular core; there is impairment in cell maturation; cell polarity is lost and mitotic activity is more frequent.

Poorly-differentiated tumors (grade 3) are classified as high grade papillary urothelial carcinomas. These tumors have prominent pleomorphism of nucleus, high nucleus/cytoplasm rate, disruption in all epithelium layers and very frequent mitotic activity.

Until the 2004 classification is verified with more clinic studies, tumors should be graded with both 1973 and 2004 classifications.

1.1.7 Histological Staging of the Bladder Tumors

The tumor staging is crucial for the determination of the prognosis and the right treatment option. It is necessary to stage the tumor, in other words to investigate the localisation of the tumor, to determine whether there is an infiltration to the bladder wall or not, if so to determine the deepness of the infiltration, and to understand whether there is local and/or distant metastase or not (Figure 3). The beginning of the tumor staging is the transuretelial resection of the tumor. With the resection, the tissue required to determine the deepness of the invasion of the tumor is collected. There is a very significant relationship

between the deepness of the bladder tumor invasion and prognosis and survival (Hasui *et al.*, 1994).

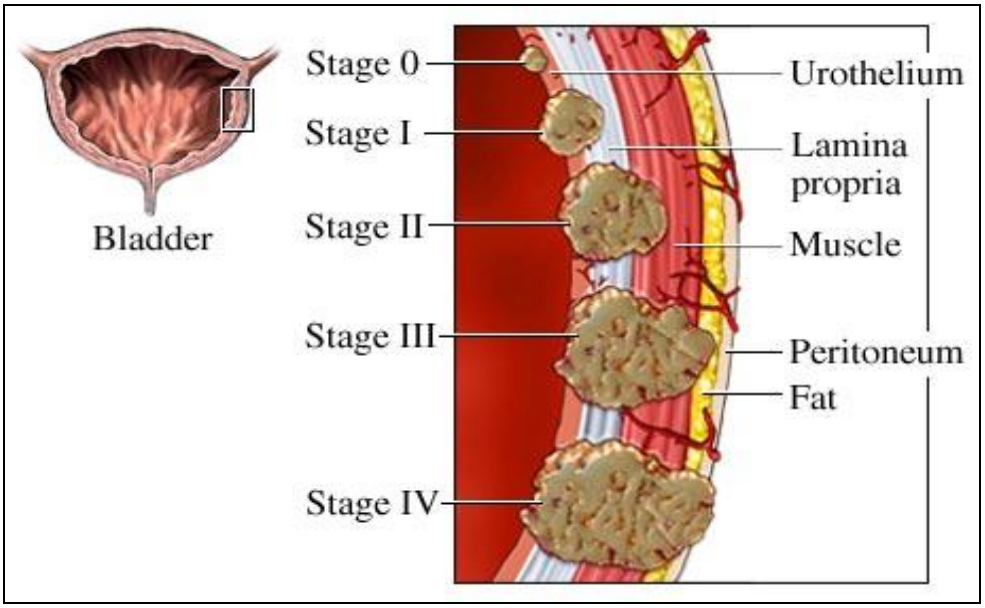


Figure 3: Stages of the bladder tumor (www.nucleusinc.com).

For staging, TNM classification of "Union International Contre le Cancer" (UICC) is widely accepted.

The letter T is followed by a number from 1 to 4 to describe how far the tumors has grown through the bladder wall and into nearby tissues. Higher T numbers mean more extensive growth.

The letter N is followed by a number from 0 to 3 to indicate any cancer spread to lymph nodes near the bladder. Lymph nodes are normally bean-sized collections of immune system cells that help fight infections and cancers.

The letter M is followed by 0 or 1 to indicate whether or not the cancer has spread to distant sites (such as other organs or lymph nodes that are not near the bladder).

Once a patient's T, N, and M categories have been determined, this information is combined in a process called stage grouping to find the cancer stage. Bladder cancer stages range from the number 0 to the Roman numeral IV. Stage 0 is the earliest and least serious stage, while Stage IV is the most advanced and serious.

T: bladder tumor categories

Bladder cancer can affect many areas of the bladder at the same time. If multiple cancers are found, the letter m is added to the appropriate T category.

Ta: Noninvasive papillary carcinoma
Tis: Carcinoma in situ (CIS); noninvasive flat carcinoma
T1: The tumor has grown below the urothelial lining but not as deep as the muscle tissue
T2: The tumor has grown into the muscle layer
T3: The tumor has grown outside the bladder into the fatty tissue that surrounds the bladder
T4: The tumor has spread to beyond the fatty tissue. It is growing into any of the following: prostate, uterus, vagina, pelvic wall, or abdominal wall

N: regional lymph node categories

This category only describes the lymph nodes near the bladder (the regional lymph nodes). These are the lymph nodes that are in the true pelvis. Any other lymph nodes are considered distant lymph nodes (the M category is used for spread to distant nodes). Surgery is usually needed to find cancer spread to lymph nodes, since it is not often seen on imaging studies.

NX: Regional lymph nodes cannot be assessed due to lack of information
N0: No regional lymph node spread
N1: The cancer has spread to a single lymph node that is 2 cm (4/5 inch) or smaller
N2:

*The cancer has spread to a single lymph node that is larger than 2 cm but not larger than 5 cm (2 inches); or
*The cancer has spread to 2 or more lymph nodes, none of which is larger than 5 cm
N3: The cancer has spread to a lymph node that is larger than 5 cm

M: distant spread (metastasis) categories

MX: Distant spread was not determined
M0: No signs of distant spread
M1: The cancer has spread to distant lymph nodes, organs, or tissues (like the bones, lungs, or liver)

1.1.8 Symptoms of Bladder Cancer

Bladder cancer may be diagnosed incidentally or because of symptoms. The most common symptom of bladder cancer is painless and episodic hematuria which is seen in 85% of patients. On the other hand, in all patients who are diagnosed with tumor during cystoscopy have microscopic hematuria (Messing *et al.*, 1990). In some patients, irritative symptoms are present from the very beginning. The symptom complex which consists of the irritability of the bladder, frequent urination, urgency and disuria is the second most common symptom and it is thought to be related with carcinoma in situ or invasive bladder cancer (Kirkali *et al.*, 2005). Incidental cancer is usually found on ultrasound performed for hematuria or irritative symptoms or for screening of symptoms unrelated to the urinary tract.

1.1.9 Diagnosis of Bladder Cancer

1.1.9.1 Imaging

1.1.9.1.1 Intravenous pyelogram

An intravenous pyelogram (also known as IVP, pyelography, intravenous urogram or IVU) is a radiological procedure used to visualize abnormalities of the urinary system, including the kidneys, ureters, and bladder. This procedure is most common for patients who have unexplained microscopic or macroscopic hematuria. It is used to ascertain the presence of a tumour or similar anatomy-altering disorders.

1.1.9.1.2 Ultrasound

Ultrasound is cyclic sound pressure with a frequency greater than the upper limit of human hearing. Its usage is increasing since it does not involve to use a contrast material which can cause allergic reactions in some patients. In addition, the usage of more sensitive transducers leads to more sharp visualisation of the urinary channel and bladder.

1.1.9.1.3 Computed Tomography

Computed tomography (CT) is a medical imaging method employing tomography. Digital geometry processing is used to generate a three-dimensional image of the inside of an object from a large series of two-dimensional X-ray images taken around a single axis of rotation. Although it gives information about primary bladder tumors, the main field of application is the scan of the metastase of the bladder tumors.

1.1.9.1.4 Magnetic Resonance

Magnetic resonance is superior to the CT in the detection of the progress of the bladder tumor to the perivesical tissue. The sensitivity of the MR in bladder tumor is 80%.

1.1.9.1.5 Skeletal scintigraphy

The importance of the routine application of the skeletal scintigraphy before total cystectomy (surgical removal of all or part of the urinary bladder) of the infiltrative tumors is still unclear except the patients with increased alkalene phosphotase level or skeletal pain.

1.1.9.2 Cytology

Cytology is the investigation of the malign cells in the bladder wash or urine sediment. Since the adhesion is weak between malign cells, they generally flow into the urine or into the bladder wash. The investigation of the cancer cells in the urine or in the bladder wash is useful in high grade malignancies and CIS. Cytology use is not cost effective except high risk population (Gamarra *et al.*, 1984).

Although the urine cytology has been a diagnostic tool commonly used in the bladder tumor diagnosis, it has 34% (20-53%) median sensitivity and 99% (83-100%) median specifity.(Lotan *et al.*, 2003). The sensitivity is even lower in low grade tumors. Therefore, while positive cytology is a contributory investigation in the diagnosis of bladder tumor, negative cytology does not exclude the possibility of low-grade bladder tumor. In addition, factors such as differences in urine collection, differences in sample processing of laboratories, urinary infection existence, urinary stone existence, intracavitary therapy and long-term catheter usage can effect the reliability of the cytology (Nabi *et al.*, 2003). There are major differences between voided urine cytology and bladder wash cytology. However, bladder wash cytology has higher diagnostic yield compaired to voided urine since it contains more superficial cells (Murphy *et al.*, 1984).

The bladder wash sample can also be used with flow cytometry. It is a technique that measures DNA amount in the cells which are stained by fluorescent dyes which can bind to DNA. Flow cytometry measures the amount of aneuploid cells. For this purpose, bladder instillation (bladder washing) sample is used. The cells with diploid DNA generally mean low grade & stage tumors. The cells

with triploid and tetraploid DNA generally mean high grade & stage tumors with worse prognosis. If aneuploidy is observed in more than 15% of the cells, this is significant for carcinoma (Güney *et al.*, 2001).

1.1.9.3 Urine-Based Techniques

1.1.9.3.1 BTA Test

This test detects from the urine the human comlement factor H-related protein (CFRhrp) which is produced by the bladder cancer cell. It gives the result in short time. It has 58% median sensitivity and 73% specifity (Van Rhijn *et al.*, 2009) (Table 2). The sensitivity is even lower in low grade tumors (Table 3).

1.1.9.3.2 Nuclear Matrix Protein (NMP)

It measures the specific protein BLCA-4 which regulates the mitosis and chromatin. This protein is expressed from the mucosa with normal appearance when there is bladder tumor or directly from the tumor. It is not expressed in the absence of the tumor. It is available as a quantitative enzyme-linked immunosorbent assay (ELISA) or, recently, as a POC test (BladderCheck) with 10 IU/ml as the threshold for a positive result. NMP22 ELISA has 69% median sensitivity and 73% specifity (Van Rhijn *et al.*, 2009) (Table 2). NMP22 POC test, on the other hand, has 62% median sensitivity and 86% specifity (Van Rhijn *et al.*, 2009) (Table 2). The sensitivity is even lower in low grade tumors (Table 3).

1.1.9.3.3 BLCA-4

BLCA-4 is a nuclear transcription factor present in both the tumor and adjacent benign areas of the bladder but not in benign bladders. BLCA-4 is measured in the urine using ELISA. Randomized trials are needed to further study their usefulness on a larger scale. It has 89% sensitivity and 95% specifity (Van Le *et al.*, 2005).

1.1.9.3.4 UBC(Monoclonal Urinary Bladder Cancer) Test

UBC-Rapid tests detects the presence of cytokeratin 8 and 18 in the urine of the bladder cancer patients. It has 35.6% sensitivity and 75% specifity (Schroeder *et al.*, 2004).

1.1.9.3.5 CYFRA 21-1 Test

It detects the cytokeratin 19 in the urine of the bladder cancer patients. It has 67.3% sensitivity and 88.4% specifity (Sánchez-Carbayo *et al.*, 2001).

1.1.9.3.6 Aura Tek Fibrin Degradation Product (AuraTek FDP)

The Aura Tek FDP Test is based on the principle that blood cells as well as plasma proteins such as fibrin are constantly shed from bladder tumors. This is caused by the effect of vascular growth factors produced by the tumor cells. Aura Tek FDP Test is an ELISA test that detects FDPs in the urine. It has 68% sensitivity and 96% specifity (Schmetter *et al.*, 1997).

1.1.9.3.7 TRAP (Telomeric Repeat Amplification Protocol) Test

It measures the telomerase activity with PCR. Specific repeats of oligonucleotides at the ends of chromosomes (telomeres) are shortened by cell division in somatic cells and are thought to be related to aging. Immortal cells such as germ-line cells or cancer cells have demonstrated increased activity of the telomere-elongating enzyme (telomerase). It has a sensitivity of 35% in voided urine and 50% in bladder wash (Dalbagni *et al.*, 1997).

1.1.9.3.8 HA (Hyaluronic acid), Hyaluronidase, HA-Haase Test

Hyaluronidase is a class of extracellular matrix-degrading endoglycosidases that regulates tumor invasion, metastasis and angiogenesis. It degrades hyaluronic acid (HA), a free nonsulfated glycosaminoglycan. The limited degradation of HA by hyaluronidase results in the generation of HA fragments of specific lengths that are angiogenic. Urinary HA and hyaluronidase are measured using ELISA-like techniques. It has a sensitivity of 91% in voided urine and 84% in bladder wash (Tinzl *et al.*, 2003).

Table 2: Median sensitivity and specificity and number of studies, institutions, and patients for five common bladder tumor detection techniques and cytology (data apply to patients under surveillance for bladder cancer) (Van Rhijn *et al*., 2009).

Marker	Studies/institute (*n*/*n*)	No. pts.	Sensitivity	Range (min–max)	No. pts.	Specificity	Range (min–max)
BTA stat	17/14	1377	58	29–74	2084	73	56–86
NMP22 Elisa	16/14	1018	69	47–100	1325	73	55–98
NMP22 POC	2/2	159	62	50–86	654	86	77–87
uCyt + /ImmunoCyt	8/6	626	77	52–100	2109	74	62–82
FISH UroVysion	6/7	256	65	30–86	311	83	66–95
Microsatellite	7/4	192	73	58–92	884	76	73–100
Cytology	26/20	2213	35	13–75	3322	94	85–100

Pts. = patients; POC = point-of-care.

Table 3: Median sensitivity per grade (G1-3, WHO 1973) and specificity of five common bladder tumor detection techniques (Van Rhijn *et al*., 2009).

Marker (number of studies)	No. pts./sensitivity			No. pts./specificity
	G 1	G 2	G 3	
BTA stat (7)	228/45	206/60	208/75	972/79
NMP22 Elisa (4)	111/43	139/58	144/82	357/64
NMP22 POC (1)	38/32	16/44	32/75	565/87
uCyt + /ImmunoCyt (3)	172/79	108/86	113/90	1509/72
FISH UroVysion (3)	52/38	28/51	38/82	169/75
Microsatellite (6)	69/61	53/63	40/92	869/77
Cytology (10)	239/17	274/34	201/58	861/95

Pts. = patients; POC = point-of-care.

1.1.9.4 Cell-Based Techniques

1.1.9.4.1 Quanticyt System

Quanticyt is an automated karyometric image analysis system that evaluates nuclear shape and DNA content of exfoliated cells obtained from bladder washings. It then is able to assign a low-, intermediate-, or high-risk "score" for bladder cancer. Since a relatively large number of cells are needed, the test requires catheterization (which is a process allowing the patient's urine to drain freely from the bladder to a plastic tube known as a urinary catheter for collection, or to inject liquids used for treatment or diagnosis of bladder conditions) for a bladder wash specimen,. The test is unlikely to become widely available because it requires technical expertise and expensive equipment. It has 59% sensitivity and 93% specifity (Wiener *et al.*, 2009).

1.1.9.4.2 uCyt+ Test

uCyt combines cytology with an immunofluorescence assay (immunocytochemistry). uCyt detects cellular markers for bladder cancer in exfoliated urothelial cells using 3 fluorescent monoclonal antibodies to pinpoint a high-molecular-weight form of carcinoembryonic antigen and 2 bladder tumor cell-associated mucins. Because the test requires the use of a fluorescence microscope by trained personnel, it is performed in a reference laboratory. It has 77% median sensitivity and 74% specifity (Van Rhijn *et al.*, 2009) (Table 2).

1.1.9.4.3 Urovision

Multiple chromosomes, such as 1, 3, 4, 7, 8, 9, 11, and 17, are altered in urothelial tumors. These chromosomal alterations can be easily detected with fluorescence in situ hybridization (FISH) assay. UroVysion is a multitarget FISH assay that detects aneuploidy in chromosomes 3, 7, and 17 as well as loss of the 9p21 locus using a fluorescence microscope.

As a result, the investigation of new tumor markers, is an attractive subject in the early bladder tumor detection, follow-up and in the detection of the prognosis.

Although the combined usage of some tests with urine cytology can be useful, none of the tests is 100% sensitive and specific (Table 2). Because of the problems faced in the standardisation of these tests and the limitations in their productions, their routine usage is still not suitable. Therefore until a 100% sensitive and specific test is found, none of these tests is accepted as an alternative to the cystoscopy and cytology by the clinicans and pathologists. Therefore, cystoscopy combined with urine cytology still retains its status as the gold standard in the screening and detection of the bladder tumor (Ekici *et al.*, 2005). It has 65% median sensitivity and 83% specifity (Van Rhijn *et al.*, 2009) (Table 2).

1.1.9.5 Cystoscopy

Cystoscopy is the endoscopy of the urinary bladder via the urethra . Diagnostic cystoscopy is usually carried out with local anaesthesia. General anaesthesia is sometimes used for operative cystoscopic procedures.

The cystoscope has lenses like a telescope or microscope. These lenses let the doctor focus on the inner surfaces of the urinary tract. Many cystoscopes have extra tubes to guide other instruments for surgical procedures to treat urinary problems.

There are two main types of cystoscopy - flexible and rigid - differing in the flexibility of the cystoscope. Flexible cystoscopy is carried out using local anaesthesia on both sexes. Rigid cystoscopy can be performed under the same conditions, but is generally carried out under general anaesthesia, particularly in male subjects, due to the pain caused by the probe.

Other than monitoring, doctor should collect sample for the urine cytology during the cystoscopy. The bladder washing sample is taken by placing an isotonic solution into the bladder (through a tube) and then by removing for microscopic testing. It’s delivered through a catheter.

Bladder cancer has the highest recurrence rate of any malignancy. Therefore, the follow-up of these individuals by the control cystoscopy, usually at 6-month intervals is very important. However, cystoscopy is an invasive diagnostic tool that impair patient comfort and also it is relatively expensive (Lotan *et al.*, 2002; Botteman *et al.*, 2003). In fact, because of the lifelong need for monitoring for recurrence with cystoscopy and for treatment of recurrent tumors, the cost per patient with bladder cancer from diagnosis to death is the highest among all cancers ($96,000 to $187,000 per patient in the United States) (Botteman *et al*., 2003).

1.1.10 Treatment of The Bladder Tumors

The treatment of the bladder tumor depends on the stage and grade of the tumor. Therefore, the treatment of the bladder tumor is 3 types: Treatment of the superficial tumors (Ta, T1, Tis), invasive tumors (T2, T3, T4) and metastatic tumors.

1.1.10.1 Treatment of the superficial tumors

1.1.10.1.1 Transuretelial resection (TUR)

It is generally first treatment in the bladder tumors. With TUR, the histologic diagnosis of the tumor is done, information about the invasion deepness and degree is obtained. For the low stage tumors, only resection is enough to treat. Cystoscopy should be done before TUR; starting from penile urethra, bladder neck, trigone and orifices should be observed. Bladder should be voided with either sterile water or mannitol in order to see around the tumor clearly. Sampling should be done from the bladder bottom and also muscle sample should be collected since the muscle invasion changes the treatment. In addition, sampling from the bladder side walls, upper wall and prostatic urethra is advised. In this way, dysplasia and carcinoma in situ existence can be understand.

1.1.10.1.2 Laser Therapy

For this purpose neodymium:YAG, holmium:YAG and potassium titanyl phosphate (KTP) lasers are used. The advantage is the absence of obturator reflex and the bleeding. The disadvantages are the absence of sampling and the cost.

1.1.10.1.3 Intravesical Therapies

Although TUR is generally enough to treat, 60-70% of the superficial tumors recur. The purpose of the intravesical therapy is to decrease the recurrence, prevent progression and destroy post-TUR tumors. Ideally, agent should be cheap, one dose and the side effects should be minimum. However there is not such an agent.

Most common agents which have being used are: thiotepa, doxarubicin, epirubicin, valrubicin, mitomycin c, bacille calmette-guerin (BCG), etoglusid.

1.1.10.1.4 Follow-up

Superficial bladder tumor patients should be followed once in every 3 months in the first year, once in every 6 months in the second year and then once every year. The cystoscopy is the golden standard in the follow-up and the urine cytology should be used in correlation.

1.1.10.2 Treatment of Invasive Tumors

In the muscle invasive tumors, there are two approach as bladder protective and radical operative. The bladder protective therapy is the combination of TUR with chemotherapy or radiotherapy. However treatment success is not high. The most common alternative used by the urologists is the radical cystectomy.

1.1.10.2.1 Radical cystectomy

This is the most suitable therapy for the patients with muscle invasive tumor. Since the disection of the lymph node and cystectomy is applied, the exact stage of the tumor can be understood. In radical cystectomy, bladder, reginal lymph

nodes in men prostate, seminal vesicals, in women uterus, cervix, vagin front wall are removed. 5 years survival rate is 50-60%.

1.1.10.2.2 Partial cystectomy

It is suitable for small, muscle invasive tumors. In addition, the place of the tumor should be suitable for co-removal with the bladder wall. Even though the capacity decreases, the patient lives with his/her own bladder.

1.1.10.2.3 Chemotherapy

In the patients with muscle invasive tumor, chemotherapy is sometimes given before radical operation in order to make the tumor smaller and to lower the stage. The patients who take chemotherapy, go to the radical cystectomy later than the patients who do not take chemotherapy. However there is still not a proof of positive effect on the survival.

1.1.10.3 The Treatment of Metastatic Tumors

Sometimes bladder tumor can be too aggressive. In spite of the treatment, metastase can happen. Also the first diagnose can be the metastatic tumor. In this case cystectomy is not the treatment which has to be chosen. In metastatic tumor, cisplatin based chemotherapy regimens are used. Systemic chemotherapy regimens are difficult regimens regarding side effects. 5-years survival rate is only 5% in patients who take chemotherapy. Radiotherapy can be used in patients who have skeletal metastase or in the case of bleeding.

1.2 Electromagnetic Radiation

All electromagnetic radiation has fundamental properties and behaves in predictable ways according to the basics of wave theory.

Electromagnetic radiation consists of an electrical field (E) which varies in magnitude in a direction perpendicular to the direction in which the radiation is traveling, and a magnetic field (M) oriented at right angles to the electrical field (Figure 4). Both these fields travel at the speed of light (c).

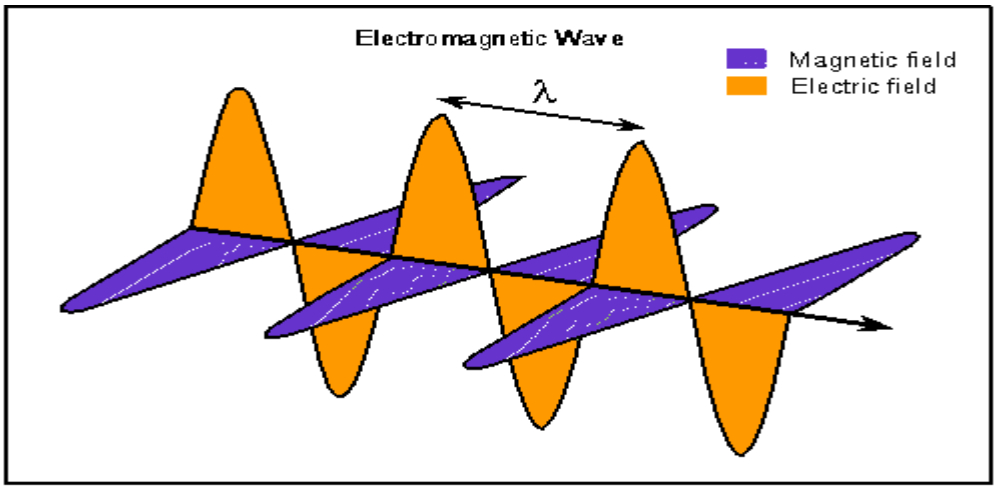


Figure 4: Electrical field and magnetic field (www.astronomynotes.com).

Two characteristics of electromagnetic radiation are the wavelength and frequency. Wavelength and frequency are related by the following formula:

$$c = \lambda \nu$$

where:

λ = wavelength (m)
ν= frequency (cycles per second, Hz)
c =speed of light (3×10^8 m/s)

The wavelength (λ) is the length of one wave cycle, which can be measured as the distance between successive wave crests. Wavelength is measured in metres (m) or some factor of metres such as nanometres (nm, 10^{-9} m), micrometres (μm, 10^{-6} m) or centimetres (cm, 10^{-2} m). Frequency refers to the number of cycles of a wave passing a fixed point per unit of time. Frequency is normally measured in hertz (Hz), equivalent to one cycle per second, and various multiples of hertz.

The interaction of light with matter can take some different forms: scattering (i.e. its direction of propogation changes), absorbtion (i.e. its energy is transferred to the molecule) or emission (energy is released by the molecule). When the energy of the light is absorbed, the molecule is said to be *excited.* An excited molecule can possess any one of a set of discrete amounts (quanta) of

energy described by the laws of quantum mechanics. These amounts are called the energy levels of the molecules.

The total energy of a molecule consists of distinct reservoirs of energy and is given by:

$$E_{total} = E_{rotation} + E_{vibration} + E_{electronic} + E_{translation} +$$

$$E_{electron\ spin\ orientation} + E_{nuclear\ spin\ orientation}$$

Each E in the equation represents the appropriate energy as indicated by its subscript. The separations between the neighboring energy levels corresponding to $E_{rotation}$, $E_{vibration}$ and $E_{electronic}$ are associated with the microwave, infrared and ultraviolet-visible region of the electromagnetic spectrum, respectively (Campbell *et al.*, 1984). The means of study for these energy transitions is standard absorption spectroscopy for electronic transition, infrared and Raman spectroscopy for vibrational (Figure 5) and rotational transitions and nuclear magnetic resonance for nuclear spin orientation and electron spin resonance for electron spin orientation (Freifelder, 1982).

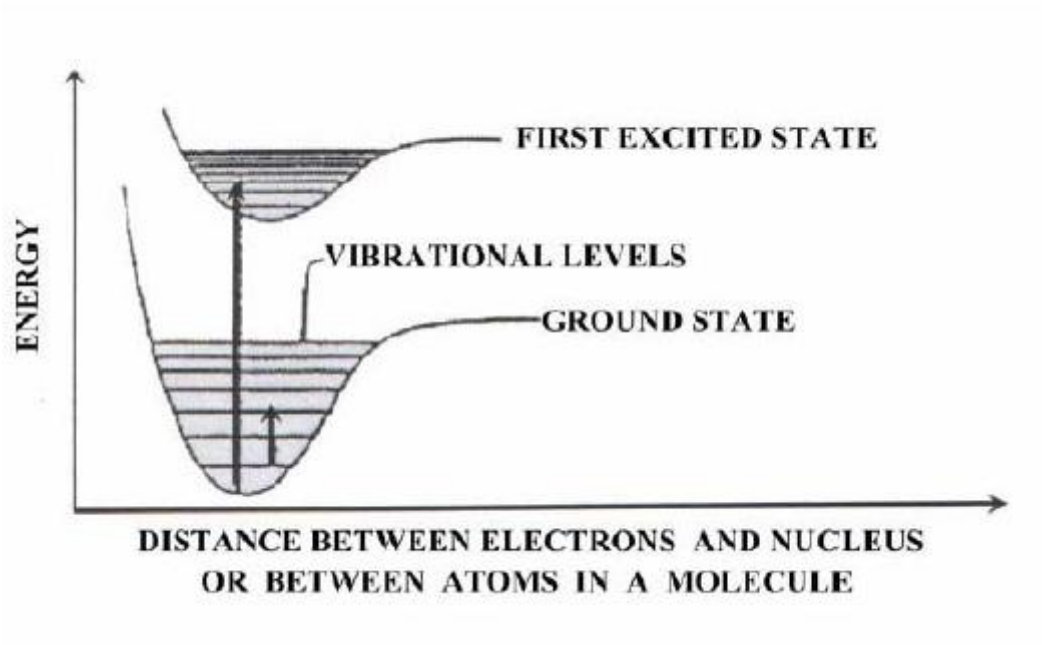


Figure 5: Typical energy-level diagram showing the ground state and the first excited state. Vibrational levels are shown as thin horizontal lines. A possible electronic transition between the ground state and the fourth vibrational level of the first excited state is indicated by the long arrow. A vibrational transition within the ground state is indicated by the short arrow (Freifelder, 1982).

1.3 Infrared Spectroscopy

1.3.1 Basis of infrared (IR) Spectroscopy

Spectroscopy is defined as the study of interaction of electromagnetic radiation with matter, excluding chemical effects. A plot of the energy absorbed ($E = h\nu$) as a function of wavelength—or more commonly frequency is referred to as a spectrum.

The electromagnetic spectrum ranges from the shorter wavelengths (including gamma and x-rays) to the longer wavelengths (including microwaves and broadcast radio waves) (Figure 6).

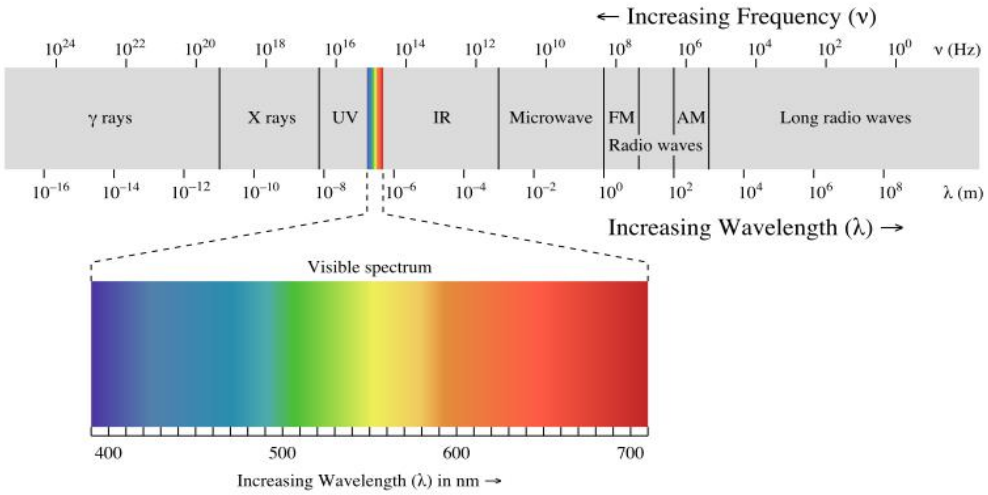


Figure 6: The electromagnetic spectrum(http://en.wikipedia.org).

Spectroscopic techniques involve irradiation of a sample with some form of electromagnetic radiation, measurement of the scattering, absorption, or emission in terms of some measured parameters, and the interpretation of these measured parameters to give useful information. Figure 6 represents many of the important regions of the electromagnetic spectrum.

Energy can reside in molecules in a number of forms, of which the most important ones, are translational, electronic, vibrational, and rotational energy. Infrared spectroscopy is based on molecular vibrations and monitors the transition between vibrational energy level (Figure 5) (Wilson *et al.*, 1995).

IR spectroscopy is the measurement of the wavelength and intensity of the absorption of infrared light by a sample. Infrared light gives energy to excite molecular vibrations to higher energy levels (Campbell *et al.*, 1984). IR radiation does not have enough energy to induce electronic transitions as seen with UV. Absorption of IR is restricted to compounds with small energy differences in the possible vibrational and rotational states.

Infrared (IR) region is divided into three sub regions (Smith, 1999):

Region	Wavenumber Range (cm^{-1})
Near-IR	14000 - 4000
Middle-IR	4000 - 400
Far-IR	400 - 4

Most infrared applications employ the mid-infrared region, but the near and far infrared regions can also provide information about certain materials. The vibrational levels and hence, infrared spectra are generated by the characteristic twisting, bending, rotating and vibrational motions of atoms in a molecule.

For a molecule to absorb IR, the vibrations or rotations within a molecule must cause a net change in the dipole moment of the molecule. The alternating electrical field of the radiation (electromagnetic radation consists of an oscillating electrical field and an oscillating magnetic field, perpendicular to each other) interacts with fluctuations in the dipole moment of the molecule. If the frequency of the radiation matches the vibrational frequency of the molecule then radiation will be absorbed, causing a change in the amplitude of molecular vibration.

Molecular rotations

Rotational transitions are of little use to the spectroscopist. Rotational levels are quantized, and absorption of IR by gases yields line spectra. However, in liquids or solids, these lines broaden into a continuum due to molecular collisions and other interactions.

Molecular vibrations

The positions of atoms in a molecules are not fixed; they are subject to a number of different vibrations. Vibrations fall into the two main catagories of *stretching* and *bending* (Figure 7).

Stretching: Change in inter-atomic distance along bond axis . A stretch is a rhythmic movement along the line between the atoms so that the interatomic distance is either increasing or decreasing. A stretch can be symmetric or asymmetric.

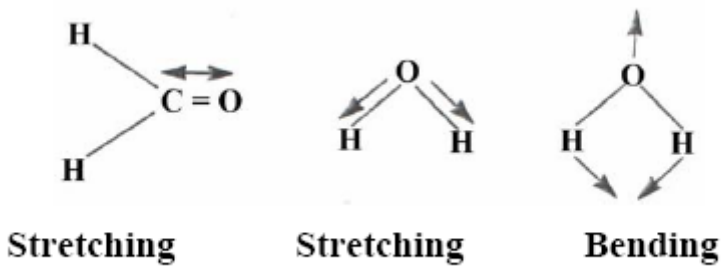


Figure 7: Stretching and bending vibrations

Bending: Change in angle between two bonds. Bending can occur in the plane of the molecule or out of plane.There are four types of bend (Figure 8):

- Rocking
- Deformation
- Wagging
- Twisting

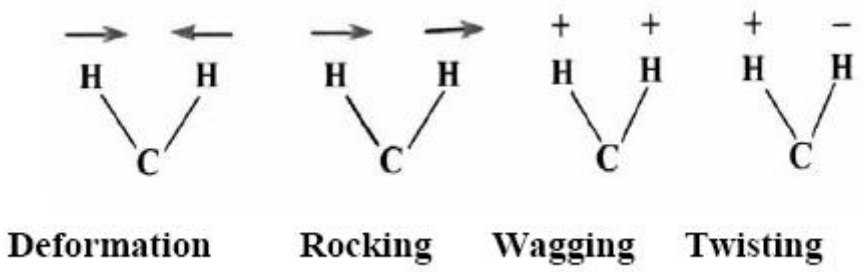


Figure 8: Types of bending vibrations

Vibrational coupling

In addition to the vibrations mentioned above, interaction between vibrations can occur (*coupling*) if the vibrating bonds are joined to a single, central atom. Vibrational coupling is influenced by a number of factors;

- Strong coupling of stretching vibrations occurs when there is a common atom between the two vibrating bonds
- Coupling of bending vibrations occurs when there is a common bond between vibrating groups
- Coupling between a stretching vibration and a bending vibration occurs if the stretching bond is one side of an angle varied by bending vibration
- Coupling is greatest when the coupled groups have approximately equal energies
- No coupling is seen between groups separated by two or more bonds

Consequently, infrared spectra are generated by the characteristic motions of various functional groups (e.g.methyl, carbonyl, amide etc.). The sensitivity of these modes of vibration to any alteration in chemical structure, conformation, and environment presents the value of infrared spectroscopy.

1.3.2 Fourier-Transformed Infrared (FT-IR) Spectroscopy

Fourier transform infrared (FT-IR) spectroscopy is a valuable analytical technique which studies molecular changes in various biological samples. It detects molecular changes from the vibrational changes in the functional groups of many organic and inorganic compounds.

This method is based on the idea of the interference of radiation between two beams to yield an interferogram, which is a signal produced as a function of the change of pathlength at a beam splitter. The most important feature of an interferogram is that every individual data point of this signal contains information over the entire infrared region. This process is carried by an interferometer. It encodes the initial frequencies into a special form, which the

detector can observe. For rapid-scanning interferometers liquid nitrogen cooled mercury cadmium telluride (MCT) detectors are used. For slower scanning types of interferometer, pyroelectric detectors (e.g. a deuterated triglycine sulfate (DTGS) detector element) can be used. In essence, the detector is always observing all frequencies at the same time (Griffiths *et al.*, 1986). The two domains of distance and frequency are interconvertible by the mathematical method of Fourier transformation. Therefore, Fourier transformation is simply a mathematical means of sorting out the individual frequencies for the final representation of an infrared spectrum.

The basic components of an FTIR spectrometer are shown schematically in Figure 9. The radiation emerging from the source is passed through an interferometer to the sample before reaching a detector. Upon amplification of the signal, in which, high frequency contributions have been eliminated by a filter, the data are converted to a digital form by an analog-to-digital converter and then transferred to the computer for Fourier transformation to be carried out.

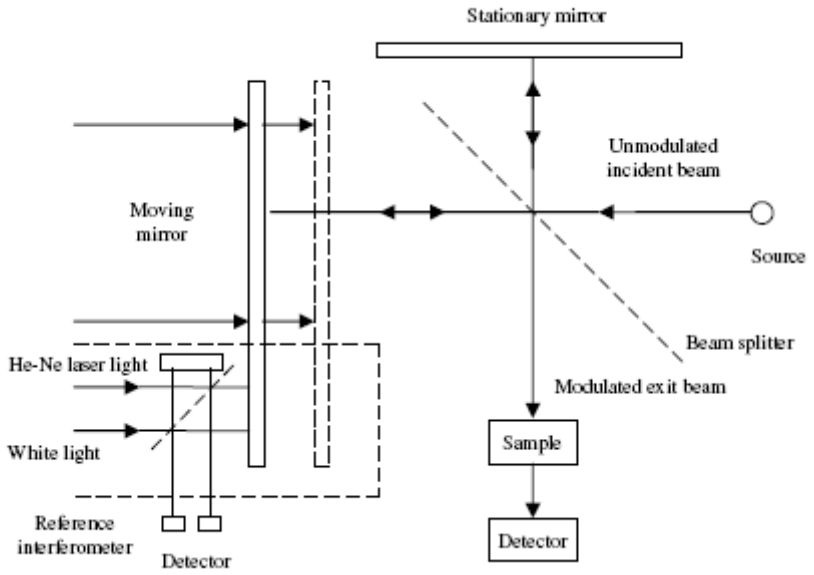


Figure 9: The Components of FT-IR spectroscopy

1.3.3 Advantages of FT-IR Spectroscopy

- FT-IR spectroscopy can be applied to the analysis of any kind of material. Samples may be solutions, viscous liquids, suspensions, inhomogeneous solids or powders (Colthup *et al.*, 1975).
- FT-IR spectroscopy is a very precise measurement method, which does not require external calibration. In addition, it is rapid, sensitive, and easy to perform (Rigas *et al.*, 1990, Manoharan *et al.*, 1993, Ci *et al.*, 1999).
- It is a non-destructive technique (Melin *et al.*, 2000; Severcan *et al.*, 1999; Cakmak *et al.*, 2003).
- The instrument components are easy to use and data processing is simple with the computer softwares. In addition, system permits permanent data storage, manipulation of data and quantitative calculations (Yono *et al.*, 1996, Ci *et al.*, 1999).
- Digital subtraction (that is, point-by-point subtraction of the separate spectra by a computer) can be used to produce good difference spectra. This method

has great advantages in obtaining infrared spectra in aqueous solutions (Campbell, 1984).

- Small sample quantities are sufficient to analyse and in vivo studies are possible (Mendelsohn *et al.*, 1986).
- Since a computer is already used to obtain the Fourier transform, it is easy to perform many scans to improve the signal-to-noise ratio (noise adds up as the square root of the number of scans, whereas signal adds linearly). It has dramatically improved signal to noise ratio by the averaging of numbers of scans per sample (Beaten *et al.*, 1998).
- Kinetic and time-resolved studies are possible (Mantcsch *et al.*, 1986; Mendelson *et al.*, 1986; Severcan *et al.*,1999).
- Frequency and bandwidth values can be determined routinely with uncertainties of better than ± 0.05 cm^{-1}.
- The price is considerably more reasonable, especially compared to NMR spectroscopy (Diem, 1993).
- The FT-IR has advantage in terms of spectral regions which originate from molecular vibrations and different molecular moieties. For instance “head group” and “hydrocarbon tails” have spectral regions for membranes.

1.3.4 Applications of Fourier Transform Infrared Spectroscopy

In a FT-IR spectrum, the shifts in peak positions and changes in the bandwidth, area values of the bands, give valuable structural and functional information, which may have diagnostic value.

Any group or bond in a molecule gives rise to characteristic band(s) in the infrared spectra. Thus, these characteristic spectral features can simply be assigned to the particular groups or bonds in the corresponding molecules. This approach in vibrational spectroscopy is referred to as group frequencies (Diem, 1993). Hence; spectra can be examined in several groups depending on the type group frequency.

The predominant near-infrared spectral features include: the methyl C-H stretching vibrations, methylene C-H stretching vibrations, aromatic C-H stretching vibrations, N-H from primary amides, secondary amides (both alkyl, and aryl group associations), N-H from primary, secondary, and tertiary amines, and N-H from amine salts.and O-H stretching vibrations. Minor but still important spectral features include: methoxy C-H stretching, carbonyl associated C-H stretching.

FT-IR spectroscopy has been widely used in the molecular investigation and diagnosis of many diseases ranging from diabetes and malignancy to infectious diseases (Fung *et al.*, 1996; Boyar *et al.*, 2003; Dogan *et al.*, 2007; Toyran *et al.*, 2006; Severcan *et al.*, 2005; Rigas *et al.*, 1990).

In different cancer types, different tissue types have been used and the changes between the healthy and cancerous tissues have been investigated at the molecular level (Andrus *et al.*, 1998; Sahu *et al.*, 2004; Li *et al.*, 2005; Rigas *et al.*, 1990).

Especially in cancer, early diagnose is very important. Rapid detection of the tumors significantly reduce mortality and the cost associated with the treatment. Most commonly used diagnosis method in hospitals is based on the investigation of the tissue samples (biopsies) by a pathologist. Spectroscopic techniques can detect (Li *et al.*, 2005) the tumors, even classify (Andrus *et al.*, 1998) tumors rapidly, with very small sample. FTIR can detect changes towards cancer at the molecular level before it can be seen in pathology.

1.4 The aim of this study

Two methods which are golden standards in bladder tumor diagnosis are cystoscopy and cytology. However the sensitivity of the urine cytology, especially in low grade tumors, is low. On the other hand, cystoscopy is an invasive technique which disturbs the patient comfort. Another problem with these systems is that, they depend on the observer and the biopsy interpretation can be highly subjective. Also there are inconsistencies between the results of

different pathologists and even between the different results of the same pathologist.

Therefore there is a need for a new technique which can detect the tumor in early stages, does not disturb the patient comfort and is automated.

In the FT-IR studies of bladder cancer, bladder tissues and tissue cultures were used before (Matysiak *et al.*, 2006). However in none of the studies, bladder wash or urine samples has been used.

Our aim is to detect the bladder tumor recurrence by using Fourier transform infrared(FT-IR) spectroscopy as a novel method. This study is unique since it is the first one to use the bladder wash sample -the content of which is close to the urine- in the diagnosis of the bladder tumor.

Another aim of this study is to compare the efficiency of the FT-IR with urine cytology which is the golden standard.

CHAPTER 2

MATERIALS AND METHODS

2.1 Patients

A total of 71 individuals who were diagnosed with bladder tumor are recruited to this study, of whom 4 were women (5.6%) and 67 (94.4%) were men. In some of the patients the tumor has recurred and caused some cellular changes in differents levels, and in some patients no tumor recurrence has occurred. Based on these recurrences which are diagnosed by pathologic observations, patients were divided to four groups as:

- Control group (n=34) which has normal bladder epithelial tissue;
- Papilloma group (n=9) which has uretelial papilloma ;
- PUNLMP group (n=6) which has uretelial neoplasm with lower malignancy potential;
- Carcinoma group (n=22) which has low and high grade papillary uretelial carcinoma.

The groups were formed according to the histopathology results. All procedures used in the experiments were approved by the Ethics Committee of Tepecik Eğitim ve Araştırma Hastanesi, İzmir, Turkey (Ethics Committee meeting in 30.03.2007, and decision no:8).

Table 4: The case distribution and the average age of the groups

	CONTROL	PAPILLOMA	PUNLMP	CARCINOMA
SAMPLE #	**34**	**9**	**6**	**22**
AGE	**63,6±12,1**	**54,2±6,7**	**68,5±13,2**	**66,9±10,1**

2.2 Sample Preparation

For the detection of the bladder tumor recurrence and its classification by using FT-IR spectroscopy, the study consisted of the following steps. First step was to collect the bladder wash samples from the patients. During cystoscopy, bladder tissue was observed with the camera, tissue biopsy was taken from the suspected area and sent to the pathologist. Also the bladder wash sample of 10 ml was collected for cytology. This sample was centrifuged for 30 minutes at 10,000 rpm. The supernatant was removed and the pellet was used for urine cytology examination. The pellet left was stored at -80 ^{o}C until FT-IR analysis.

The biopsy and urine cytology results were obtained from the pathologist and compared with FT-IR results.

2.3 Sample Preparation for FT-IR Spectroscopy Studies

Second step was the FT-IR analysis of these samples. For this purpose, frozen samples stored at -80^{o}C, were dried in the lyophilizer (Labconco FreeZonet, Model 77520) for 12 hours to remove water. The samples were then ground in an agate mortar in order to obtain bladder wash powder. 0.4 mg of the powder was mixed with 100 mg potassium bromide KBr, and dried again in the lyophilizer for 6 h in order to completely remove any traces of water. The mixture was then subjected to a pressure of 100 kg/cm^2 (1200 psi) for 6 minutes in an evacuated die to produce a KBr pellet for use in FT-IR spectrometer. For

each patient, 3 pellets were prepared, all of which gave identical spectra. The reason of preparing 3 pellets is to minimize the measurement errors by taking the average of 3 scan.

2.4 FT-IR Data Analysis

Infrared spectra were obtained by scanning the prepared pellets with a Perkin Elmer Spectrum One Spectrometer (Norwalk, CT). Since water and carbondioxide molecules in the air affect the IR spectrum, the spectrum of air and KBr transparent disk was recorded together as background and substracted automatically by using appropriate software (SpectrumOne software).

Atmospheric vapor was also automatically subtracted. FT-IR spectra of bladder wash samples were recorded in the 4000-400cm^{-1} region at room temperature. One hundred scans were taken for each interferogram at 4 cm^{-1} resolution. Recording and analysis of the spectral data were performed using the Spectrum One software from Perkin Elmer.

According to the histopathology results, 4 groups were formed as control, papilloma, PUNLMP and carcinoma. The band positions were measured according to center of weight. The averages of the spectra belonging to the same experimental groups, baseline correction, normalization, and the band areas were obtained by using the same software program. The average spectra and normalization process were applied only for visual representation of the differences; however, for the determination of the spectral parameters and calculation of mean values and statistical analysis, each baseline-corrected original spectrum was taken into consideration.

Cluster analysis was applied that classifies objects, via a tree diagram (dendrogram) calculated using the Ward's algorithm. Constructed with the OPUS 5.5 software (Bruker Optics), the dendrogram graphically represents the cluster analysis groups. For cluster analysis, second derivatives of the spectra

were calculated and subsequently vector normalized over the investigated frequency range. Cluster analysis was applied first to distinguish between different spectra of control and carcinoma individuals and then to distinguish between control and diseased individuals. In this case, PUNLMP and papilloma patients were also used. As input data for cluster analysis, spectral distances were calculated between pairs of spectra as Pearson's correlation coefficients (Helm *et al*. 1991). Cluster analysis for separation of samples was based on the Euclidean distances. In all cases, Ward's algorithm was used for hierarchical clustering.

On the basis of the biopsy and cystoscopy results, the sensitivity and specifity of FT-IR spectroscopy were calculated in different spectral ranges. The performance of the methods used in the diagnosis of a disease can be described with the concepts of sensitivity ad specificity. The sensitivity measures the proportion of actual positives, which are correctly identified, e.g., the percentage of sick people who are identified as having the condition; and the specificity measures the proportion of negatives, which are correctly identified, e.g. the percentage of well people who are identified as not having the condition. A theoretical, optimal prediction can achieve 100% sensitivity (i.e., predict all people from the sick group as sick) and 100% specificity (i.e., not predict anyone as a sick from the healthy group).

Table 5: Definitions for sensitivity and specificity of a urine test against the gold standard

URINE TEST RESULT			
	POSITIVE	NEGATIVE	
TUMOR	A	B	SENSITIVITY= A/(A+B)
NO TUMOR	C	D	SPECIFITY= D/(C+D)

2.5 Statistics

The bands were identified and assigned for each group according to the literature. The results were expressed as mean±standard deviation. For each statistical comparison with control group (control-carcinoma, control-PUNLMP and control-papilloma), Mann-Whitney U test is used. A *p* value of less than 0.05 was considered significant (*p<0.05**, *p<0.01***, and *p<0.001****).

CHAPTER 3

RESULTS

This work was carried out to to investigate the bladder tumor recurrence and to classify the tumors using the bladder wash samples by using FT-IR spectroscopy as a novel method.

The bladder wash samples were collected during the control cystoscopy. Although the sample content is quite similar to the urine, its spectrum is not the same. The sample contains urine components such as urea, creatinine, uric acid and also different types of cells, cylinders, crystals (which are much lower in urine) and resectisol. Resectisol is an isotonic solution used to wash the bladder, and contains mannitole. In the spectrum of the bladder wash samples, it has major contribution to amide A, PO_2^- symmetric stretching and C-O bending bands (Table 6).

3.1 Molecular Investigation

3.1.1 Control and Carcinoma Patients

The existence of some infrared absorbtion bands, suggests the presence of specific groups of atoms in the system studied (Steele, 1971). Therefore it is possible to assign specific wavelength molecular absorption bands to specific vibrational modes of particular functional groups.

Figure 10 shows the representative infrared spectra of bladder wash sample of the control group in 4000-400 cm^{-1} region.

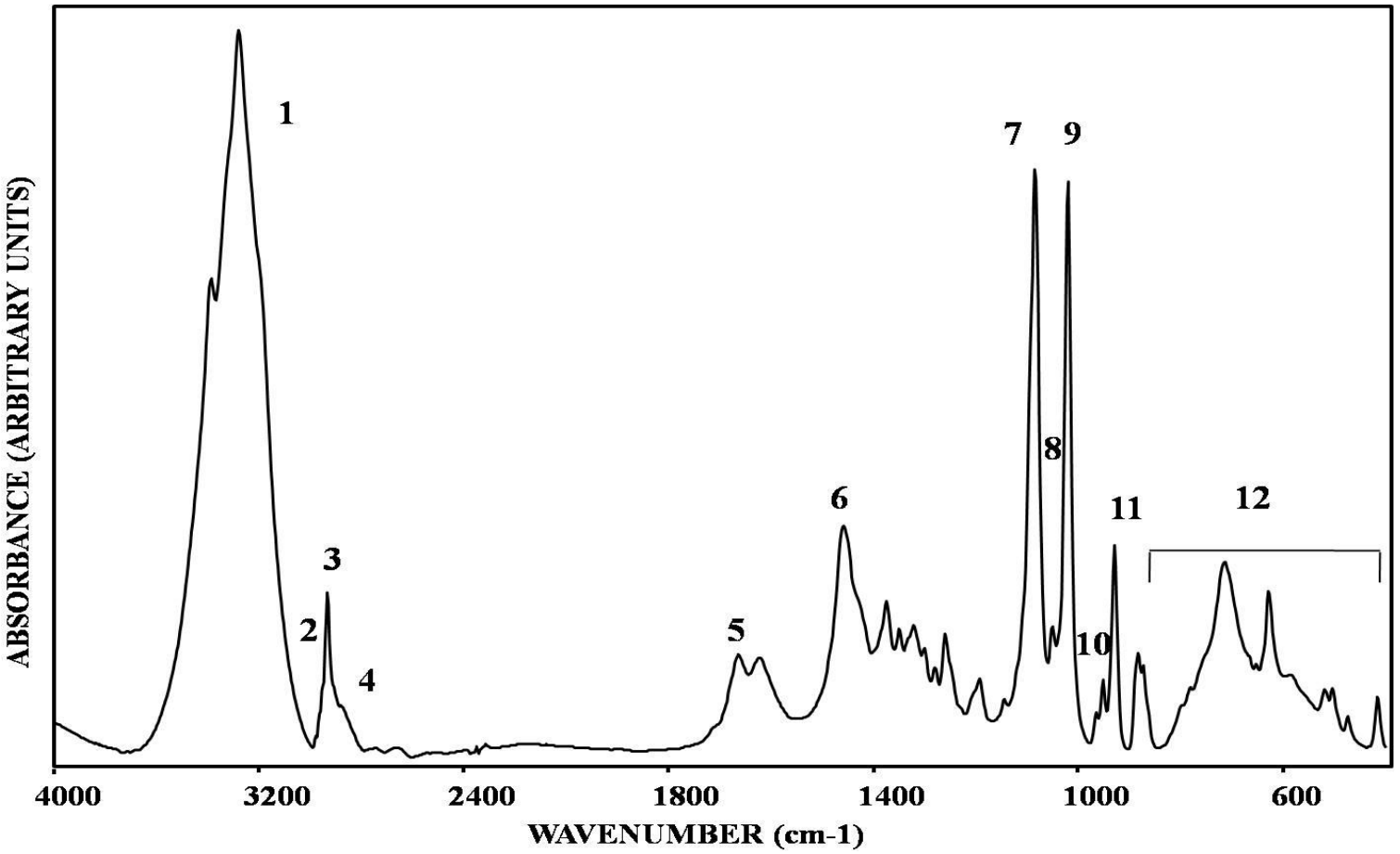


Figure 10: The representative infrared spectra of bladder wash sample of the control group in 4000-400 cm^{-1} region

The FTIR spectrum of bladder wash sample is quite complex representing many different functional groups belonging to many organic and inorganic compounds. The position and assignments of the bands were listed in Table 6. These assignments are based upon this work and other studies cited in literature (Rigas *et al.*, 1990; Wong *et al.*, 1993; Wong *et al.*, 1991; Takahashi *et al.*, 1991; Wang *et al.*, 1997; Naumann *et al.*, 1991; Jamin *et al.*, 1998; Melin *et al.*, 2000; Jackson *et al.*, 1998;; Lyman *et al.*, 1999; Chiriboga *et al.*, 2000; Cakmak *et al.*, 2003, Cakmak *et al.*, 2006; Toyran *et al.*, 2006; Raunier et al, 2003).

Normally, the urea in the urine has very dominant bands (Mansfield *et al.*, 2005). However, since the bladder wash sample is centrifuged for 30 minutes and the pellet was used in the FT-IR, the final sample does not contain as much urea, creatinine and uric acid as the original bladder wash sample.

Table 6 : General band assignment of bladder wash samples

Band Number	Frequency (cm^{-1})	Assignments
1	3282	N-H and O-H stretching
2	2958	CH_3 antisymmetric stretching
3	2936	CH_2 antisymmetric stretching
4	2877	CH_3 symmetric stretching
5	1664	C=O stretching
6	1462	C–N antisymmetric stretching
	1452	CH_2 bending
7	1086	PO_2^- symmetric stretching
8	1052	C-O stretching
9	1022	C-O bending
10	967	C-C stretching
11	930	C-O-C ring
12	840-400	Fingerprint Region

While the frequency of the bands gives information about the structure/conformation and intermolecular interactions, the area under the bands monitors the concentration of the related molecules. Therefore in order to investigate cancer induced structural and compositional variations of macromolecules in the bladder wash sample, a detailed spectral analysis was

performed in three distinct regions, namely 3795–2995 cm^{-1}, 2995-2800 cm^{-1} and 1800-400 cm^{-1} which were given in Figure 11, Figure 12 and Figure 13, respectively.

Table 7: Numerical summary of the detailed differences in the band frequencies of control and carcinoma groups spectra. The values are the mean ± standart deviation for each sample.

Frequency (cm^{-1})			
Band	**Control (n=34)**	**Carcinoma (n=22)**	**p values**
1	3282.51±13.94	3276,88±10.71	p<0.05*
2	2958.01±4.83	2959.51±0.44	p<0.001***
3	2936.76±1.61	2935.57±0.47	p<0.05*
4	2877.38±6.22	2870.55±1.47	p<0,001***
5	1664.85±1.01	1663.47±4.70	p<0.05*
6	1462.04±1.62	1462.77±0.51	p<0.001***
	1452.54±0.48	1453.04±0.62	p<0.01**
7	1086.71±1.30	1087.69±0.87	p<0.05*
8	1052.06±1.85	1053.62±0.33	p<0.001***
9	1022.62±1.46	1024.64±0.99	p<0.001***
10	967.22±0.33	967.40±0.31	p<0.05* (at limit)
11	930.92±1.32	932.30±0.39	p<0.001***
a	2914.42±3.69	2911.17±0.94	p<0.05*
c	629.44±2.32	626.62±1.66	p<0.05*

Table 8: Numerical summary of the detailed differences in the band areas of control and carcinoma groups spectra. The values are the mean ± standart deviation for each sample.

Area			
Band	**Control (n=34)**	**Carcinoma (n=22)**	**p values**
1	238.12±33.66	263.81±45.60	p<0.05*
2	2.77±0.38	3.06±0.48	p<0.05*
3	5.74±1.03	6.86±1.22	p<0.01**
4	7.04±1.35	8.27±1.22	p<0.01**
5	10.10±6.38	5.35±2.75	p<0.05*
6	18.14±1.84	20.04±2.18	p<0.01**
7	19.09±2.68	20.93±3.06	p<0.05*
8	2.08±0.30	2.52±0.42	p<0.001***
9	12.51±1.90	14.18±2.22	p<0.05*
10	0.44±0.26	0.67±0.16	p<0.01**
11	3.55±0.77	4.28±0.72	p<0.01**
b	0.93±0.90	Not observed	p<0.001***
c	5.86±0.95	5.29±0.84	p<0.05*

In Figure 11, the spectra were normalized with respect to the amide A band observed between 3795-2995 cm^{-1}. The amide A band contains strong absorptions arising from the N-H stretching modes and intermolecular O-H stretching modes (Melin *et al.*, 2000; Cakmak *et al.*, 2006). As could be seen from the figure that the control and carcinoma spectra considerably differ in peak positions in this region. Compared to control group (3282.51±13.94 cm^{-1}), there was a significant shift in the frequency of amide A to lower values (3276,88±10.71 cm^{-1}) in carcinoma group (*p<0.05**).

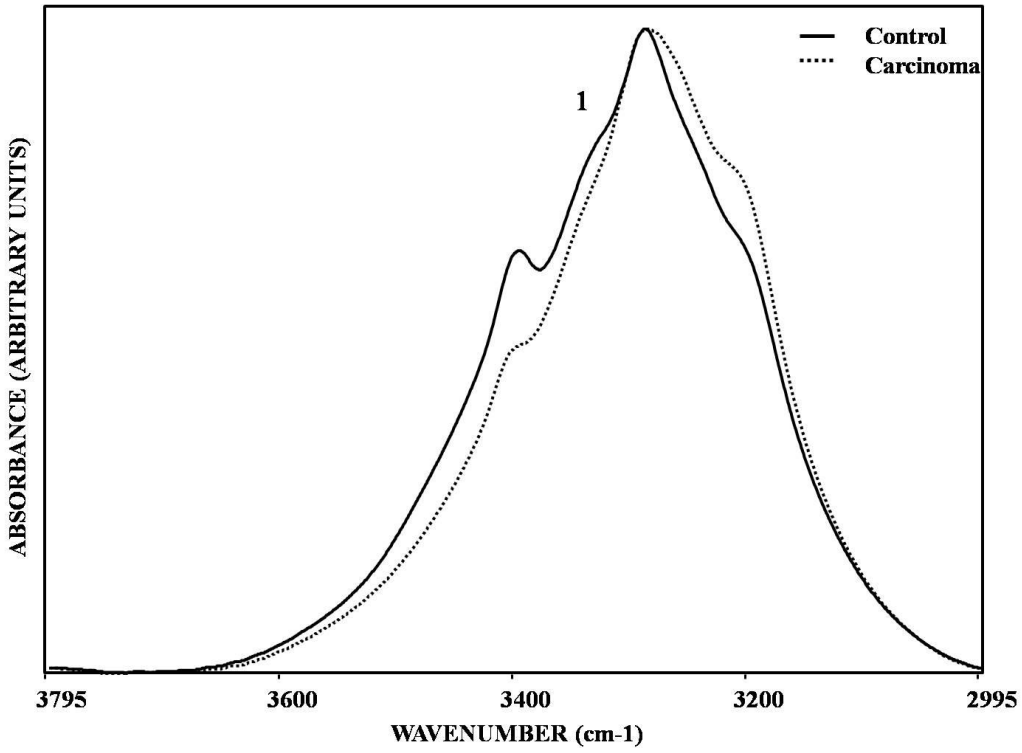


Figure 11: The average spectra of bladder wash sample of the control and carcinoma groups in 3795-2995 cm^{-1} region. The spectra were normalized with respect to the amide A band.

The representative infrared spectra of bladder wash sample of the control and carcinoma groups in 2995-2800 cm^{-1} region are shown in Figure 12. The spectra were normalized with respect to the CH_2 antisymmetric band centered around 2936 cm^{-1}. This region contains some important bands, such as the CH_3

symmetric and antisymmetric stretching modes and CH_2 antisymmetric stretching mode.

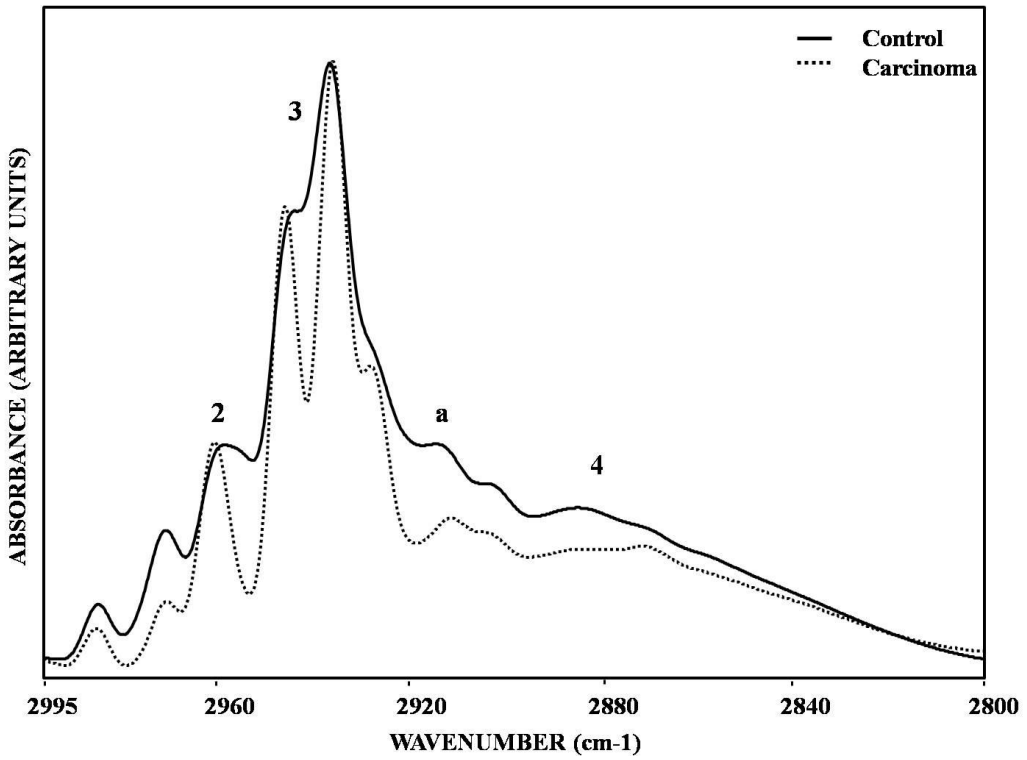


Figure 12: The average spectra of bladder wash sample of the control and carcinoma groups in 2995-2800 cm^{-1} region. The spectra were normalized with respect to the CH_2 asymmetric band.

The prominent peak at 2958 cm^{-1} is assigned to asymmetric CH_3 stretching vibrations mainly due to methyl groups of the lipids (Mantsch, 1984; Severcan *et al.*, 1997) and the shifts in the frequencies can be used as markers for the deep interior of the lipid bilayer (Umemura *et al.*, 1980). There was a significant cancer-induced shift ($p<0.001$***) in the frequency of this band from 2958.01±4.83 cm^{-1} to 2959.51±0.44 cm^{-1} (Table 7). Moreover, as can be seen in Table 8, there was a significant ($p<0.05$*) increase in the area of the band in carcinoma group.

The CH_2 antisymmetric band which is located at 2936 cm^{-1} originates mainly from methylene groups of fatty acids (Severcan *et al.*, 2003; Cakmak *et al.*, 2006). The CH_2 stretching vibrations depend on the degree of conformational disorder; hence they can be used to monitor the average *trans/gauche* isomerization in the system (Mantsch *et al.*, 1984; Severcan, 1997; Bizeau *et al.*, 2000). The frequency of the CH_2 antisymmetric band which is located at 2936.76±1.61 cm^{-1} gives information about the average *trans/gauche* isomerization in the system. This band significantly shifted ($p<0.05$*) to 2935.57±0.47 cm^{-1} (Table 7) in carcinoma group. Another difference which can be seen in Figure 12, was the significant increase ($p<0.01$**) in the area of the CH_2 antisymmetric band splitting into two at 2935 cm^{-1} and 2945 cm^{-1} in the carcinoma group. In the control group such splitting was not as prominent as the carcinoma group.

The band located at 2877 cm^{-1} is attributable to CH_3 symmetric stretching band, which is mainly due to proteins and amino acids (Melin *et al.*, 2000; Cakmak *et al.*, 2003). There was a significant shift in the frequency of this band from 2877.38±6.22 cm^{-1} to 2870.55±1.47 cm^{-1} ($p<0.001$***) in the carcinoma group (Table 7). The area of this band also significantly increased ($p<0.01$**) which suggests an increase in the protein content (Table 8).

One result that was expected but not observed was the change in the dynamics of the system which can be monitored by the bandwidth of the CH_2 asymmetric stretching mode band. In our study, there was not a significant change in the bandwidth.

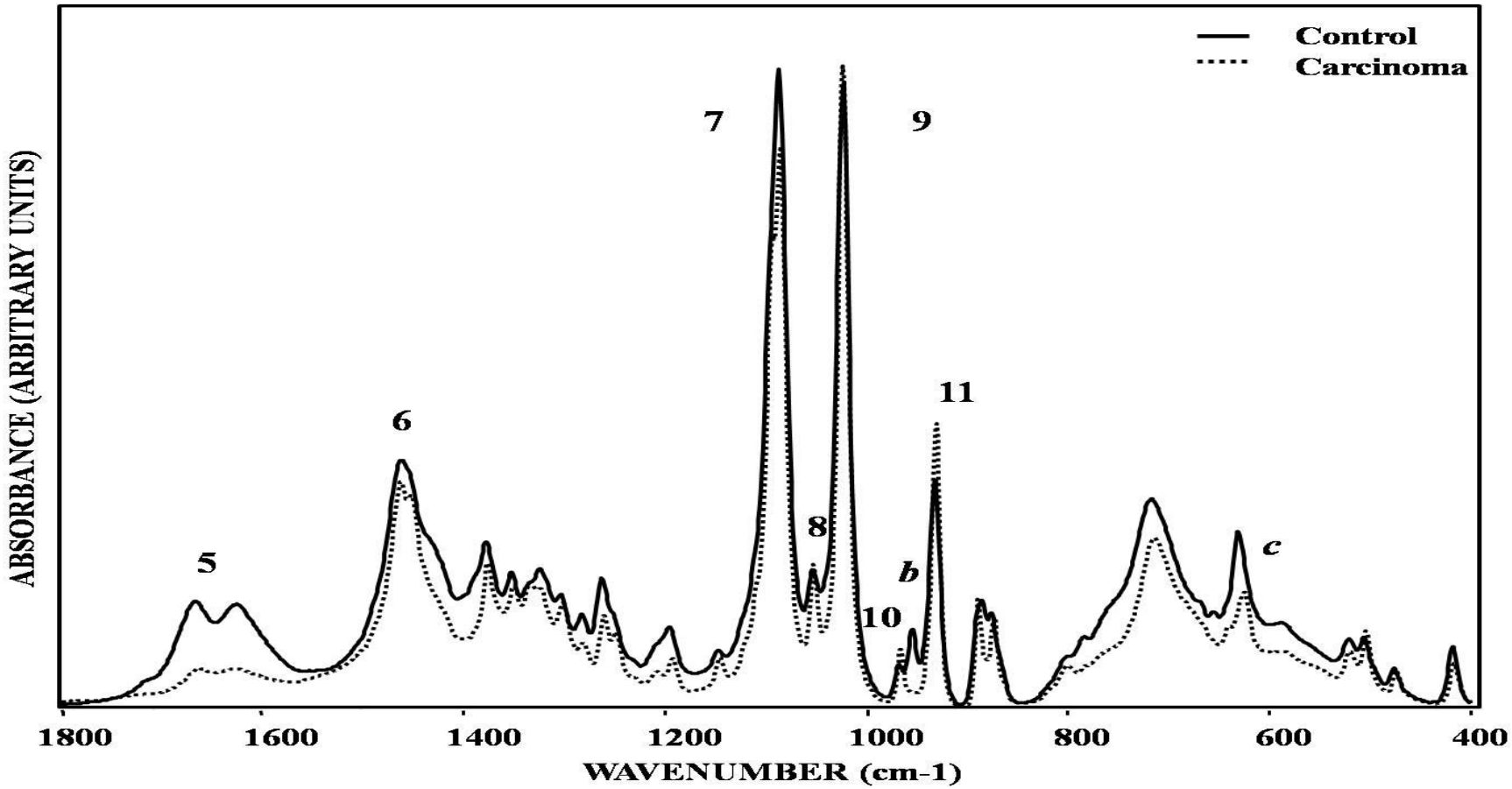


Figure 13: The average spectra of bladder wash sample of the control and carcinoma groups in 1800-400 cm^{-1} region. The spectra were normalized with respect to PO_2^- symmetric stretching band.

The band located at 1664 cm^{-1} is mainly assigned to the C=O stretching vibration. This band is generally attributed to the amide I vibration of structural proteins (Melin *et al.*, 2000; Takahashi *et al.*, 1991). However since in our study urea has a very strong contribution to this band, protein contribution to this band can be neglected. There was a significant shift in the frequency of the band from 1664.85±1.01 cm^{-1} to 1663.47±4.70 cm^{-1} ($p<0.05$*) in the carcinoma group (Table 7). The area of this band also significantly decreased ($p<0.05$*) in the carcinoma group (Table 8).

The band found at 1460 cm^{-1} is attributable to the C–N asymmetric stretching vibration which is mainly due to the urea (Raunier *et al.*, 2003). This band was overlapped with the band located at 1452 cm^{-1} which is due to the CH_2 bending of lipids (Cakmak *et al.*, 2003; Manorahan *et al.*, 1993). Therefore, to investigate the change in the area was not useful to comment on urea or lipid content. To compare these bands in the control and the carcinoma group, the second derivative spectra was studied. It is seen from the second derivative spectra (Figure 16) that there was a significant shift in the frequency of the C–N asymmetric stretching band to higher values in the carcinoma group (from 1462.04±1.62 cm^{-1} to 1462.77±0.51 cm^{-1} with $p<0.001$***). In addition, similar difference was observed in CH_2 bending vibration band (from 1452.54±0.48 cm^{-1} to1453.04±0.62 cm^{-1} with $p<0.01$**).

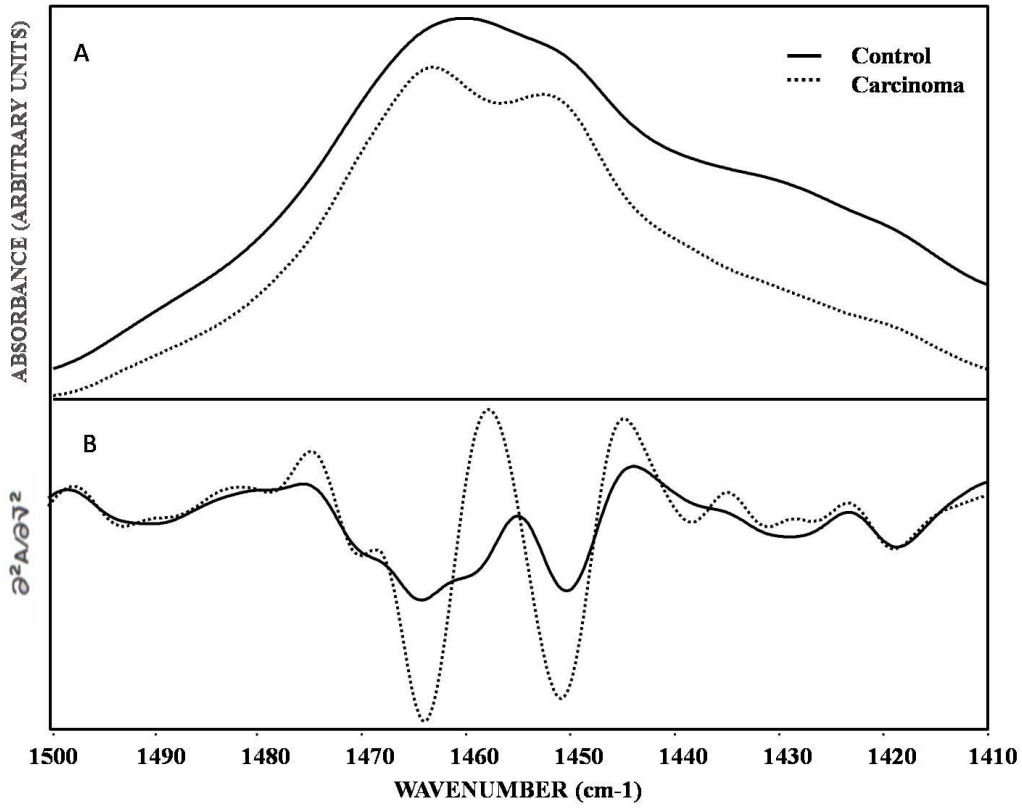


Figure 14: The average spectra of the control and carcinoma groups in 1500-1410 cm-1 region (A) Original spectra. (B) Spectra after second derivative.

The region between 900-1200 cm^{-1}, has been shown to be a major indicator of carcinogenesis (Wong *et al.*, 1991; Cohenford *et al.*, 1997; Chiriboga *et al.*, 1998). In the 1200-900 cm^{-1} frequency range, several macromolecules (e.g. polysaccharides and phosphate carrying compounds such as phospholipids and nucleic acids, give absorbtion bands (Melin *et al.*, 2000; Cakmak *et al.*, 2003). Many peaks found in this region can be used to classify the tumor.

The PO_2^- symmetric stretching band is generally assigned to be located at 1086 cm^{-1} (Lyman *et al.*, 2001; Banyay *et al.*, 2003). There was a significant shift ($p<0.05$*) from 1086.71±1.30 cm^{-1} to 1087.69±0.87 cm^{-1} in the frequency of this band in carcinoma samples (Table 7). In addition, the area of the symmetrical PO_2^- stretching band is significantly (*$p<0.05$**) increased in the carcinoma spectra as compared to the control spectra (Table 8).

The band found at 1052 cm^{-1} is attributable to C–O stretching which is mainly due to oligosaccharides and polysaccharides (Melin *et al.*, 2000; Toyran *et al.*, 2006; Cakmak *et al.*, 2006). The frequency of the C–O stretching band shifted significantly to higher values in the carcinoma group (from 1052.06±1.85 cm^{-1} to 1053.62±0.33 cm^{-1} with $p<0.001$***). Another carbohydrate band which is located at 1022 cm^{-1} is attributable to the C-O bending of the C-OH groups of carbohydrates. The frequency of the C–O bending band also shifted significantly ($p<0.001$***) from 1022.62±1.46 cm^{-1} to 1024.64±0.99 cm^{-1} in the carcinoma group. In addition, the area of the C–O stretching and C–O bending peaks increased significantly ($p<0.001$*** *and* $p<0.05$*, respectively) in the carcinoma group (Table 8).

The peak found at 967 cm^{-1} is attributable to the C–C streching vibration (Liu *et al.*, 2003) which is mainly due to DNA and can also be a good criterion to detect the tumor. There was a significant shift to higher values in the frequency of this band in the carcinoma group (from 967.22±0.33 cm^{-1} to 967.40±0.31 cm^{-1} with $p<0.05$*). Also, the area of the peak increased significantly ($p<0.01$**) in carcinoma group (Table 8). There was a significant shift ($p<0.001$***) to higher values in the frequency of the band located at 930 cm^{-1} which is also DNA band (Garip *et al.*, 2007). In addition, there was a significant increase in the area ($p<0.01$**) of this band (Table 8).

In addition to these bands whose band assignment were discussed, there are some other bands which could not be assigned. However the change in the frequency or area of these bands were significant and can be used to detect tumoral changes.

- The band located at 2914 cm^{-1} (Figure 12) in the control group shifted significantly ($p<0.05$*) to lower values in the carcinoma group (Table 7).
- The band located at 953 cm^{-1} (Figure 13) in the control group disappeared in the carcinoma group ($p<0.001$***) (Table 8).

The band located at 629 cm^{-1} (Figure 13) in the control group shifted significantly ($p<0.05$*) to lower values in the carcinoma group (Table 7). In addition the area of this band decreased significantly ($p<0.05$*) in the carcinoma group (Table 8).

3.1.2 Control and PUNLMP – Papilloma Patients

PUNLMP and papilloma groups samples showed different FT-IR spectra compared to the control samples which can be used to detect the low grade tumors. As can be seen from Figure 15, Figure 16 and Figure 17, although the differences were not as prominent as the carcinoma group, there were some bands which were significant (Table 9 and Table 10).

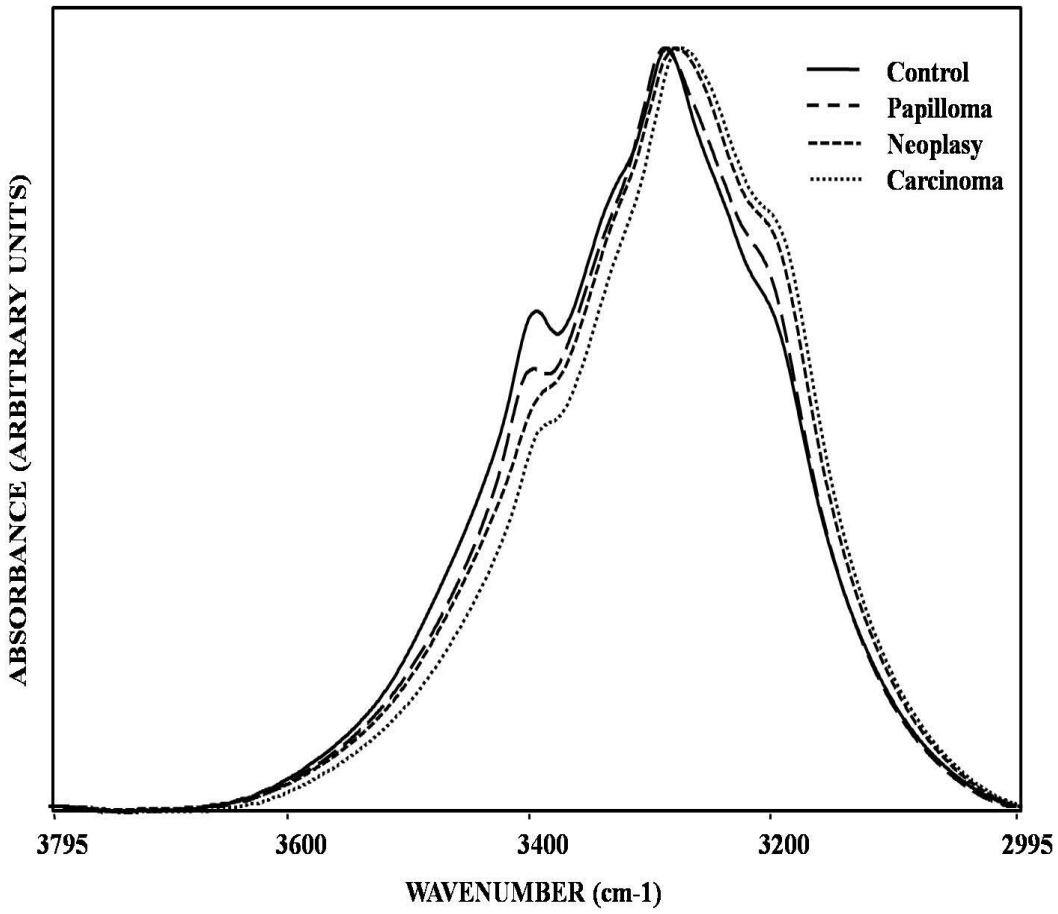


Figure 15: The average infrared spectra of bladder wash sample of the control, papilloma, PUNLMP and carcinoma groups in 3795-2995 cm^{-1} region. The spectra were normalized with respect to the amide A band.

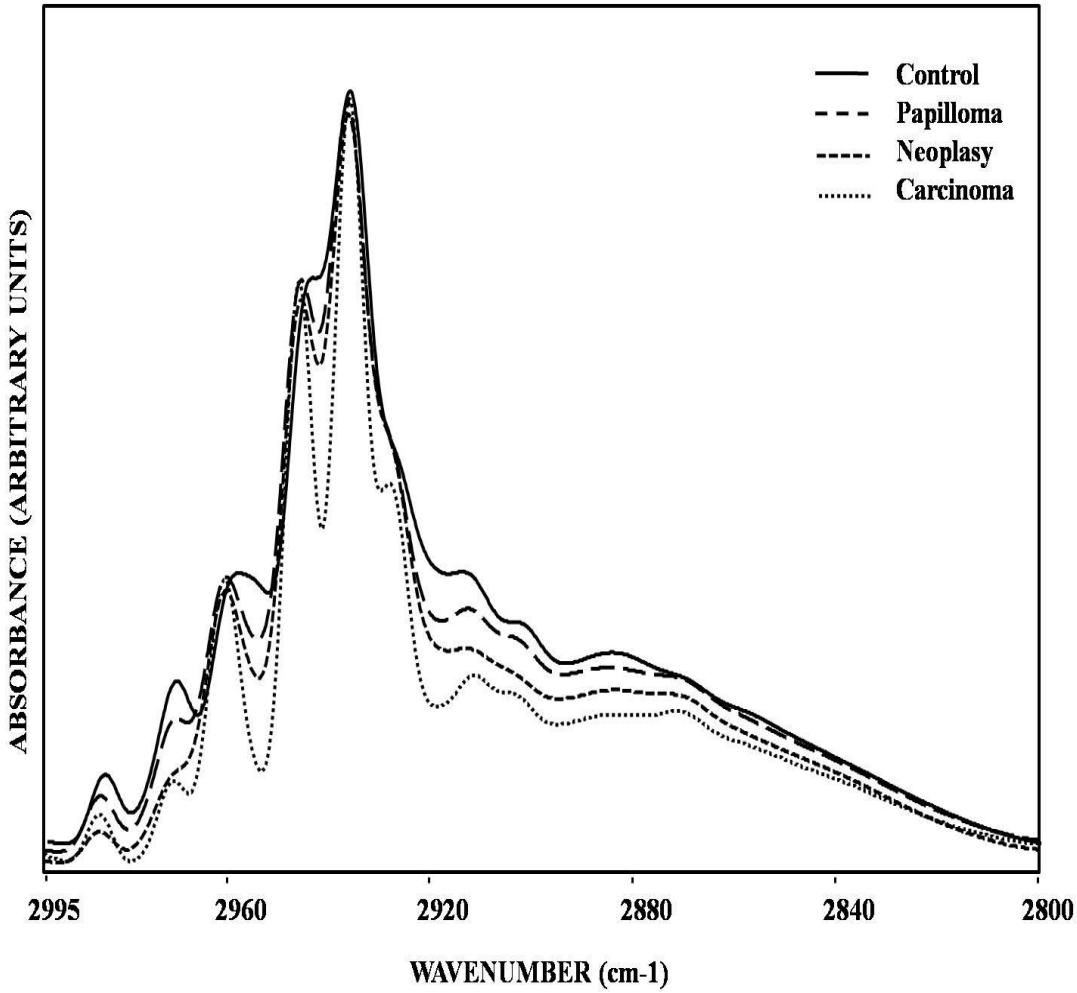


Figure 16: The average infrared spectra of bladder wash sample of the control, papilloma, PUNLMP and carcinoma groups in 2995-2800 cm^{-1} region. The spectra were normalized with respect to the CH_2 asymmetric band.

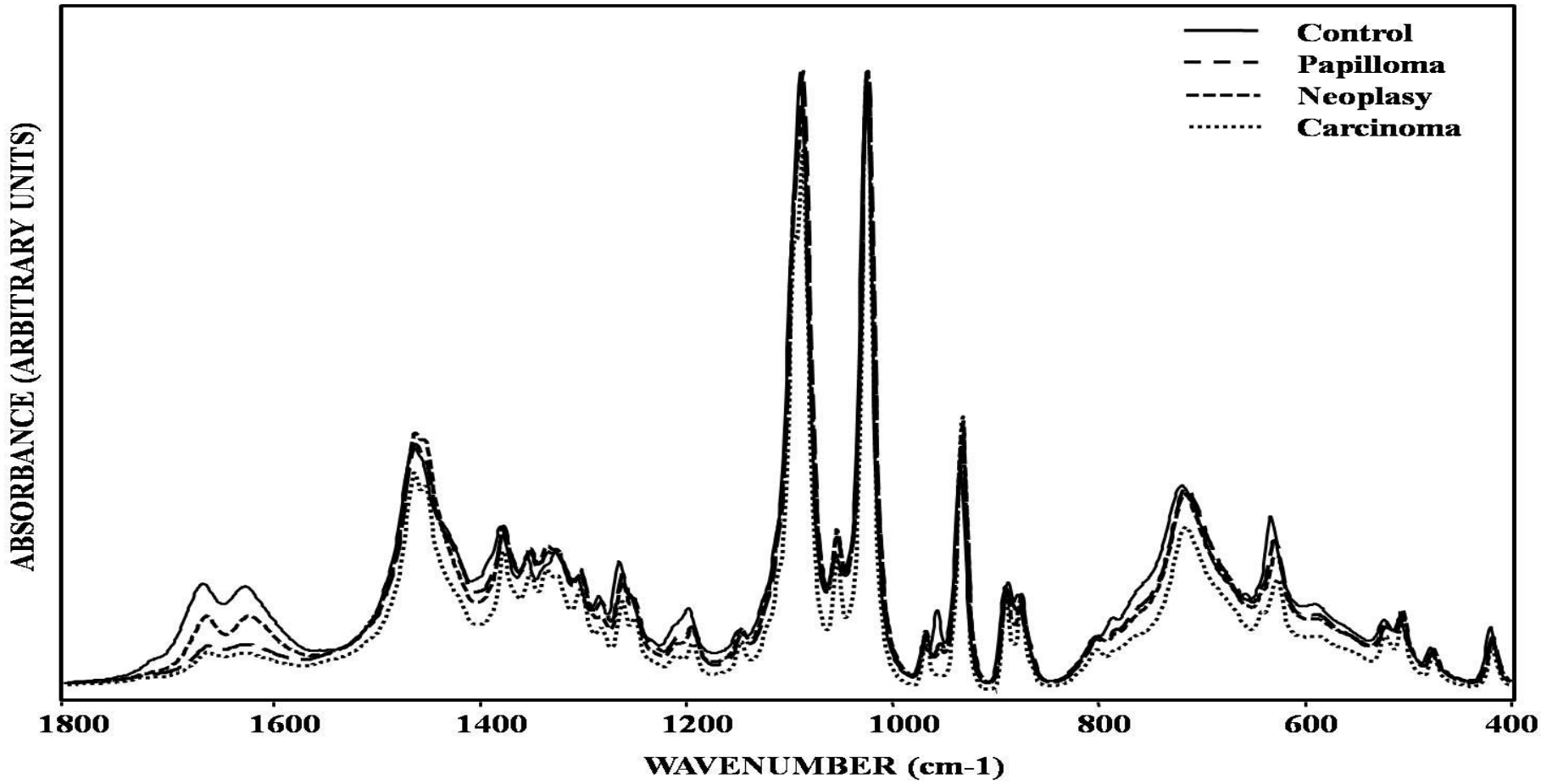


Figure 17: The average spectra of bladder wash sample of the control, papilloma, PUNLMP and carcinoma groups in 1800-400 cm^{-1} region. The spectra were normalized with respect to PO_2^- symmetric stretching band.

Table 9: Numerical summary of the significant differences in the band frequencies and areas of control and PUNLMP groups spectra. The values are the mean ± standart deviation for each sample.

Frequency(cm^{-1})			
Band	**Control(n=34)**	**PUNLMP(n=6)**	**p values**
4	2877.38±6.22	2872.36±5.75	p<0.05*
6	1452.54±0.48	1453.22±0.38	p<0.05*
8	1052.06±1.85	1053.32±0.76	p<0.05*
9	1022.62±1.46	1024.08±1.50	p<0.05*
c	629.44±2.32	626.85±0.78	p<0.05*
Area			
Band	**Control(n=34)**	**PUNLMP(n=6)**	**p values**
8	2.08±0.30	2.47±0.36	p<0.05*
c	5.86±0.95	4.88±0.39	p<0.05*

Compared to the control group, there was

- A significant (*p<0.05**) shift in the frequency of the CH_3 symmetric stretching band (band 4) to lower values (*p<0.05**) in PUNLMP group.
- A significant (*p<0.05**) shift in the frequency of the CH_2 bending band (band 6) to higher values (*p<0.05**) in PUNLMP group.
- A significant (*p<0.05**) shift in the frequency of the C–O stretching band (band 8) to higher values and an significant (*p<0.05**) increase in the area of the band in PUNLMP group.
- A significant (*p<0.05**) shift in the frequency of the C–O bending band (which is another carbohydrate band) (band 9) to higher values.
- A significant shift (*p<0.05**) in the frequency of the 629 cm^{-1} band to lower values (band c) and a significant (*p<0.05**) decrease in the area of this band in PUNLMP group.

Table 10: Numerical summary of the significant differences in the band frequencies and areas of control and papilloma groups spectra. The values are the mean ± standart deviation for each sample.

Frequency (cm^{-1})			
Band	**Control(n=34)**	**Papilloma (n=9)**	**p values**
4	2877.38±6.22	2872.96±6.03	p<0.05*
5	1664.85±1.01	1663.09±2.50	p<0.05*
6	1452.54±0.48	1453.13±0.51	p<0.05*
8	1052.06±1.85	1052.95±1.77	p<0.05*
9	1022.62±1.46	1024.02±1.30	p<0.05*
11	930.92±1.32	931.82±1.12	p<0.05*
a	2914.42±3.69	2911.00±1.22	p<0.05*
c	629.44±2.32	627.25±1.26	p<0.05*
Area			
Band	**Control(n=34)**	**Papilloma (n=9)**	**p values**
1	238.12±33.66	272.58±45.87	p<0.05* (at limit)
2	2.77±0.38	3.21±0.58	p<0.05*
6	18.14±1.84	20.67±2.64	p<0.05*
7	19.09±2.68	22.22±3.28	p<0.05*
8	2.08±0.30	2.61±0.64	p<0.05*
9	12.51±1.90	14.80±2.38	p<0.05*
b	0.93±0.90	0.37±0.75	p<0.05*

Compared to the control group, there was

- An increase in the area of amide A band (band 1) in papilloma group (*p<0.05**).
- A significant shift in the frequency of the CH_3 symmetric stretching band (band 4), which is mainly due to proteins and amino acids to lower values

($p<0.05$*) and a significant ($p<0.05$*) increase in the area of the band in papilloma group.

- A significant shift in the frequency of the C=O stretching band (band 5) to lower values ($p<0.05$*) in papilloma group.
- A significant ($p<0.05$*) shift in CH_2 bending band (band 6) to higher values and a significant ($p<0.05$*) increase in the area of the of C–N antisymmetric stretching (band 6) and CH_2 bending band (band 6) in papilloma group.
- A significant ($p<0.05$*) increase in the area of the of PO_2^- symmetric stretching band (band 7) in papilloma group.
- A significant ($p<0.05$*) shift in the frequency of the C–O stretching band (band 8) to higher values and a significant ($p<0.05$*) increase in the area of this band in papilloma group.
- A significant shift in the frequency of the C–O bending band (band 9) to higher values ($p<0.05$*) and a significant ($p<0.05$*) increase in the area of this band in papilloma group.
- A significant shift ($p<0.05$*) in the frequency of the 2914 cm^{-1} band (band a) to lower values in papilloma group.
- A significant shift ($p<0.05$*) in the frequency of the 629 cm^{-1} band (band c) to lower values and a significant ($p<0.05$*) decrease in the area of this band in papilloma group.

3.2 Cluster Analysis

As shown before, control and carcinoma samples show different FT-IR spectra. Unlike model organisms, in human studies there are differences not only "between groups" but also "within groups". In addition, in this study bladder instillation (bladder wash sample) was used and the composition of this sample is close to the urine. So there are several metabolites in the sample, and the composition of the sample can change with the diet, and many other factors. Therefore sample size and the range to be used in cluster are very important factors. There are many spectral differences. However the ranges which have statistically significant differences should be chosen for cluster analysis.

On the basis of these spectral differences, cluster analysis was performed using the bands that are significantly different between groups to differentiate between the control and carcinoma samples spectra. Hierarchial clustering of the control and carcinoma samples using second derivative spectra in the spectral ranges of 2907-2923 cm^{-1}, 1444-1457 cm^{-1}, 637-649 cm^{-1} and 625-637 cm^{-1} gave best results. The results are demonstrated in Figure 18, Figure 19, Figure 20 and Figure 21.

3.2.1 Control – Carcinoma Samples

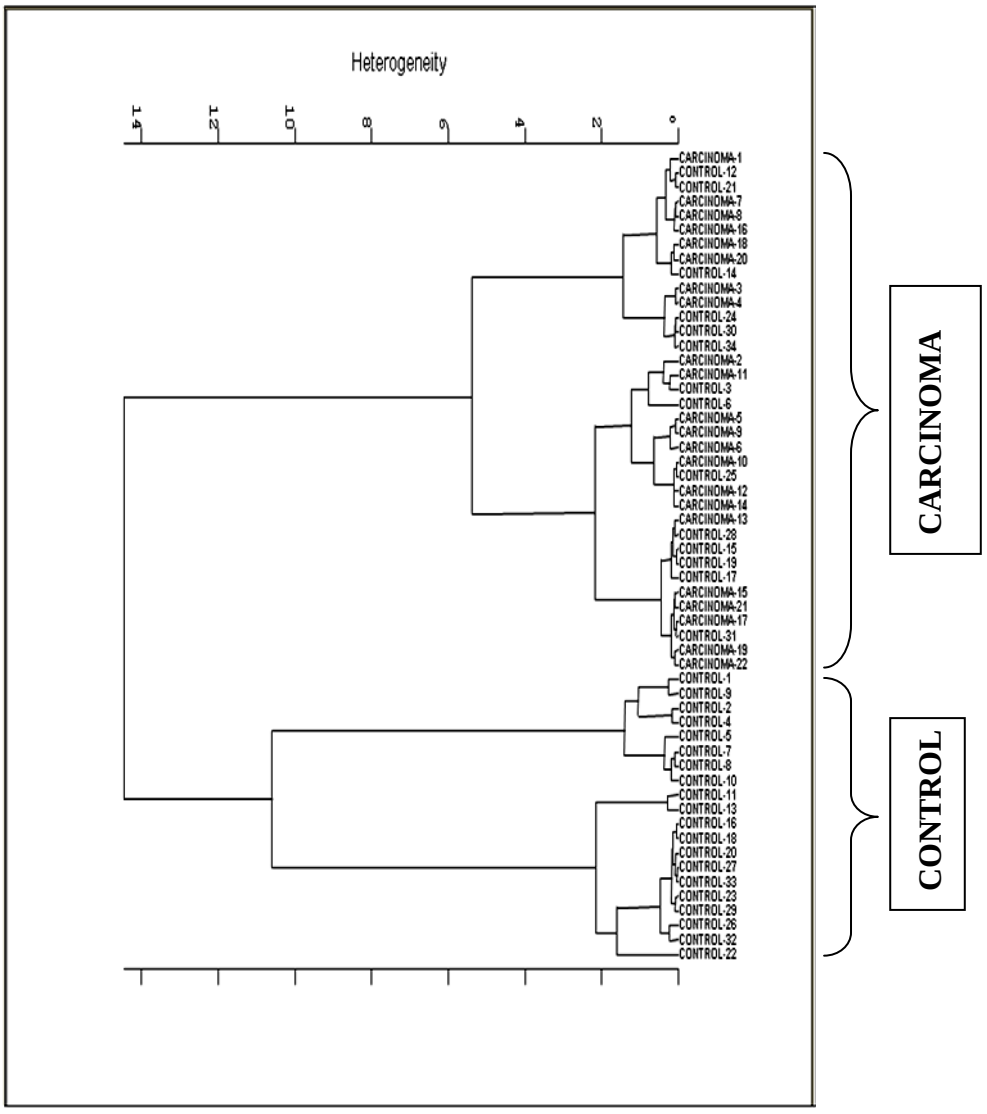


Figure 18: Hierarchial clustering of control and carcinoma samples using second derivative spectra (spectral range: 2907-2923 cm^{-1}). The sensitivity is 100% and the specifity is 58.8%.

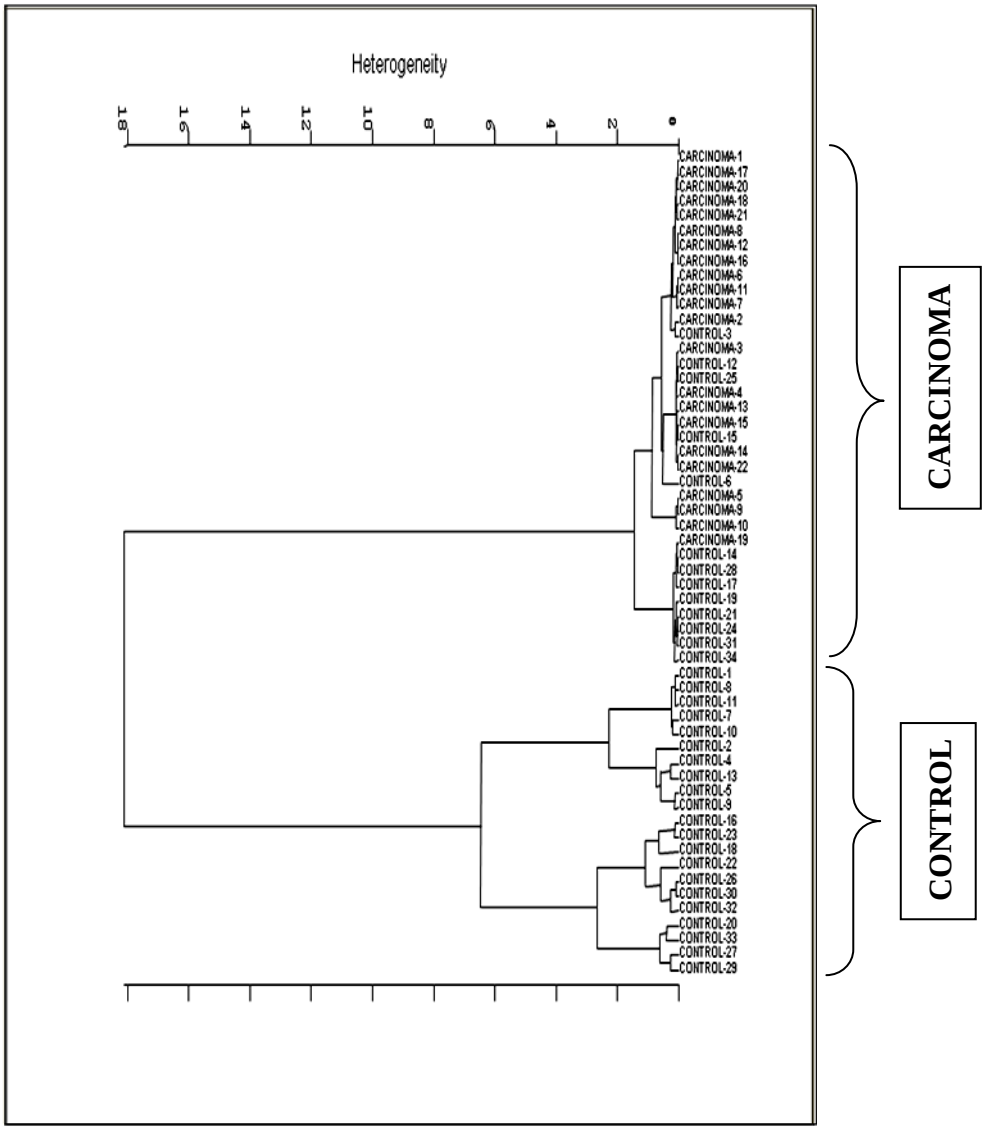


Figure 19: Hierarchial clustering of control and carcinoma samples using second derivative spectra (spectral range: 1444-1457 cm^{-1}). The sensitivity is 86.4% and the specifity is 70.6%.

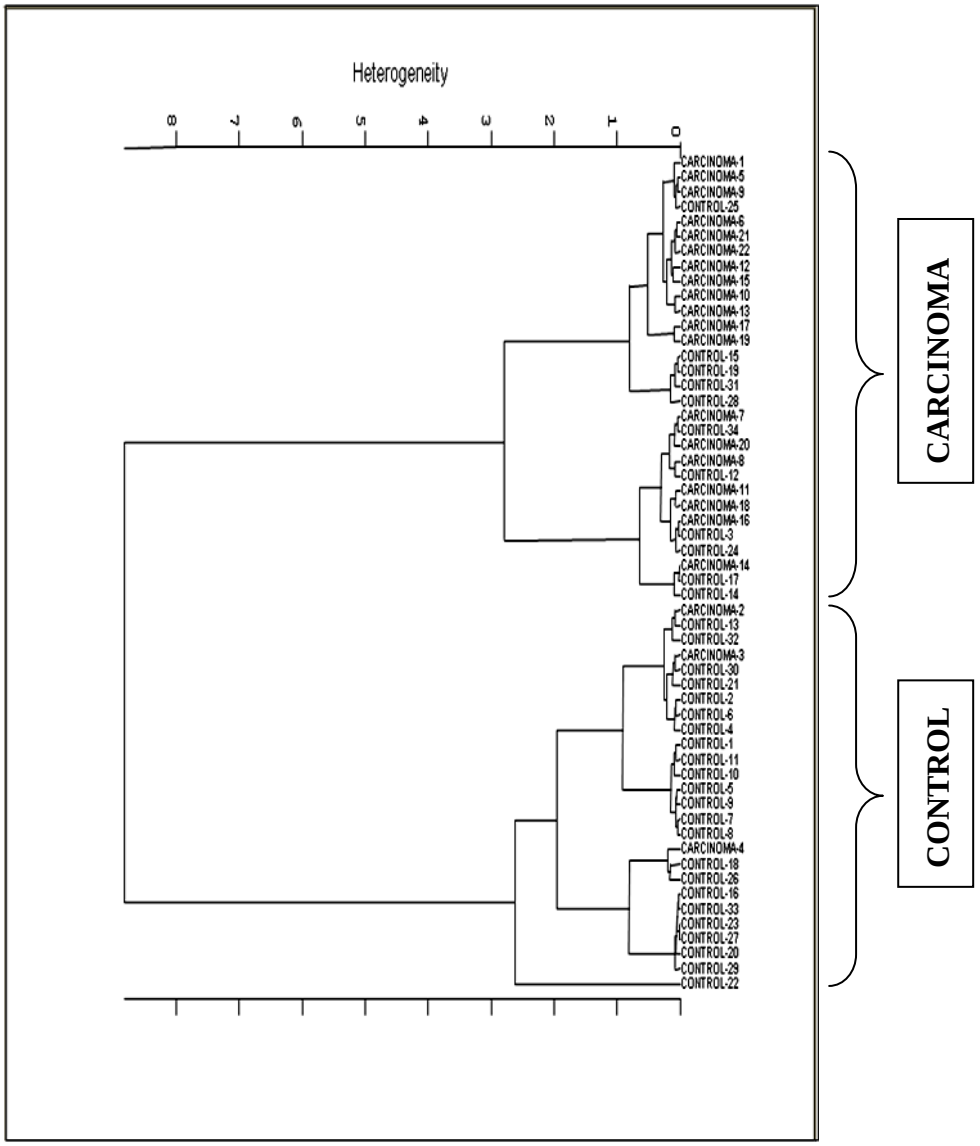


Figure 20: Hierarchial clustering of control and carcinoma samples using second derivative spectra (spectral range: 637-649 cm^{-1}). The sensitivity is 100% and the specifity is 61.8%.

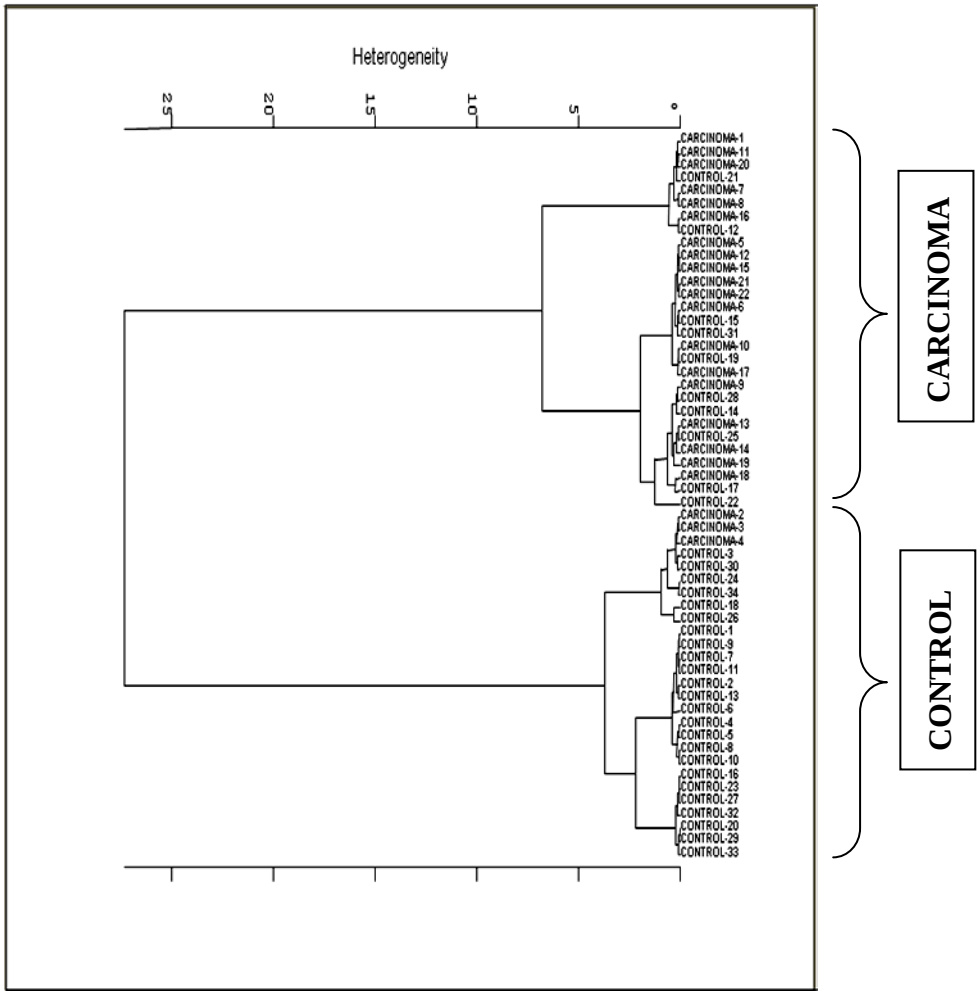


Figure 21: Hierarchial clustering of control and carcinoma samples using second derivative spectra (spectral range: 625-637 cm^{-1}). The sensitivity is 86.4% and the specifity is 70.6%.

So on the basis of the biopsy and cystoscopy results, the sensitivity and specifity of the spectral ranges were calculated. The results are seen in Table 11.

3.2.2 Control – Diseased Samples

As mentioned before, control and diseased samples show different FT-IR spectra. Since the cluster analysis discriminated the control and carcinoma samples spectra successfully, we also tried the cluster analysis of the control and diseased samples spectra. In this case, control and all tumor samples were used in cluster analysis including carcinoma, papilloma and PUNLMP samples. On the basis of these spectral differences (Table 7, Table 8, Table 9 and Table 10), cluster analysis was performed using the bands that are significantly different between the groups to differentiate between the control and diseased individuals spectra. Hierarchial clustering of the control and the diseased samples using second derivative spectra in the spectral ranges of 2954-2979 cm^{-1}, 2907-2923 cm^{-1}, 1444-1457 cm^{-1}, 1015-1033 cm^{-1}, 637-649 cm^{-1} and 625-637 cm^{-1} gave best results. The results were demonstrated in Figure 22, Figure 23, Figure 24, Figure 25, Figure 26 and Figure 27.

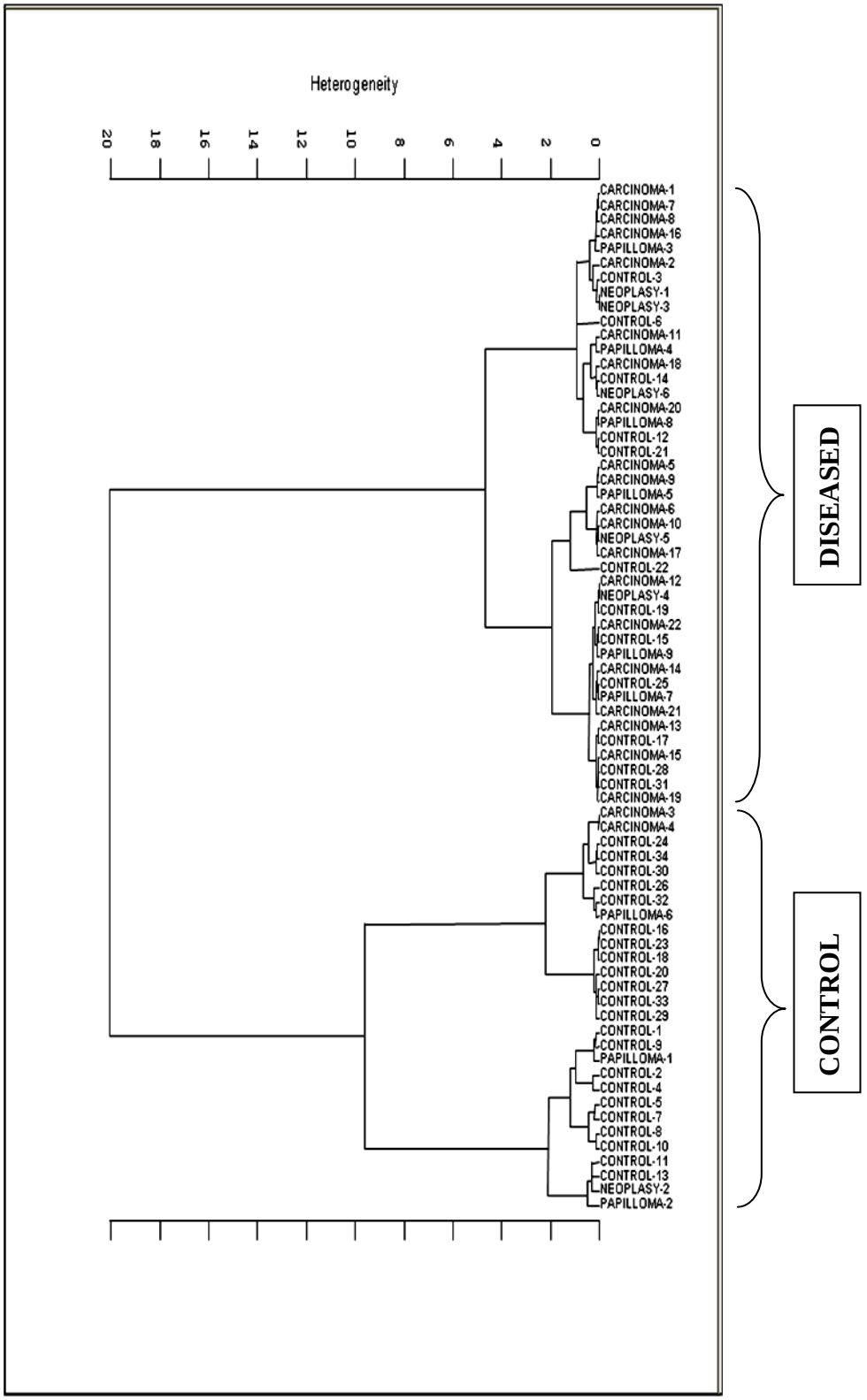


Figure 22: Hierarchial clustering of control and diseased samples using second derivative spectra (spectral range: 2954-2979 cm^{-1}). The sensitivity is 83.8% and the specifity is 67.7%.

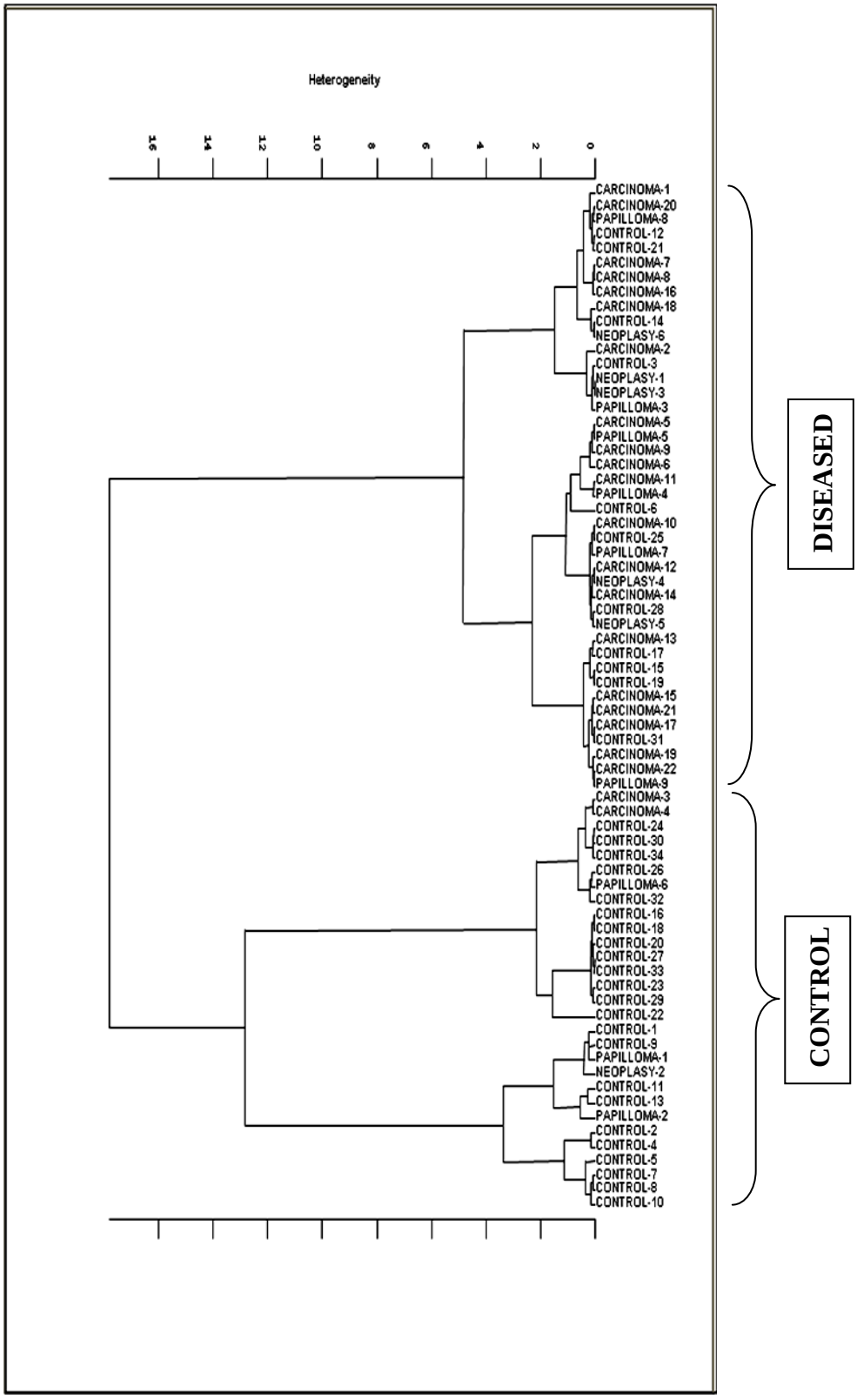


Figure 23: Hierarchial clustering of control and diseased samples using second derivative spectra (spectral range: 2907-2923 cm^{-1}). The sensitivity is 83.8% and the specifity is 67.7%.

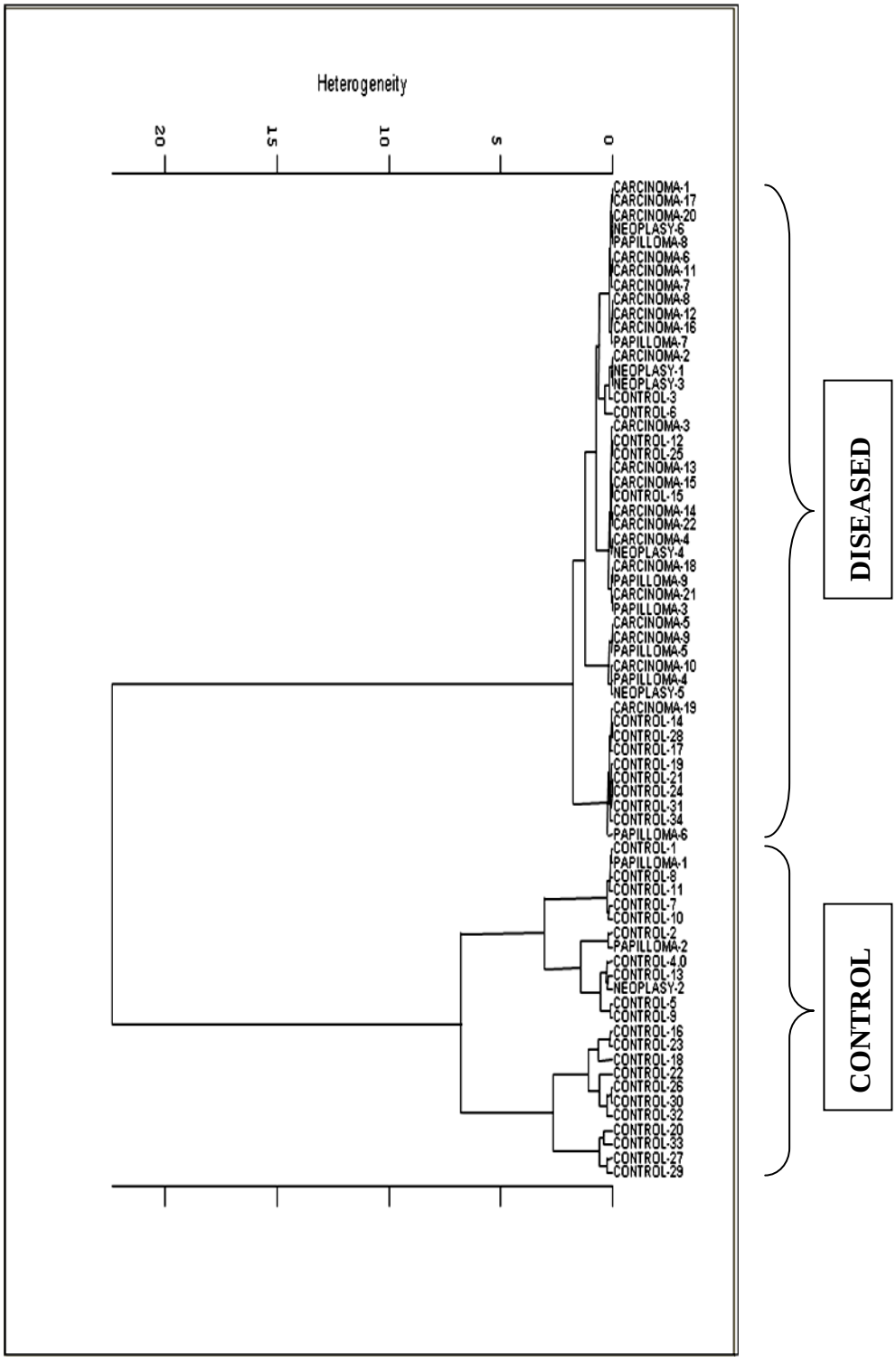


Figure 24: Hierarchial clustering of control and diseased samples using second derivative spectra (spectral range: 1444-1457 cm^{-1}). The sensitivity is 91.8% and the specifity is 61.7%.

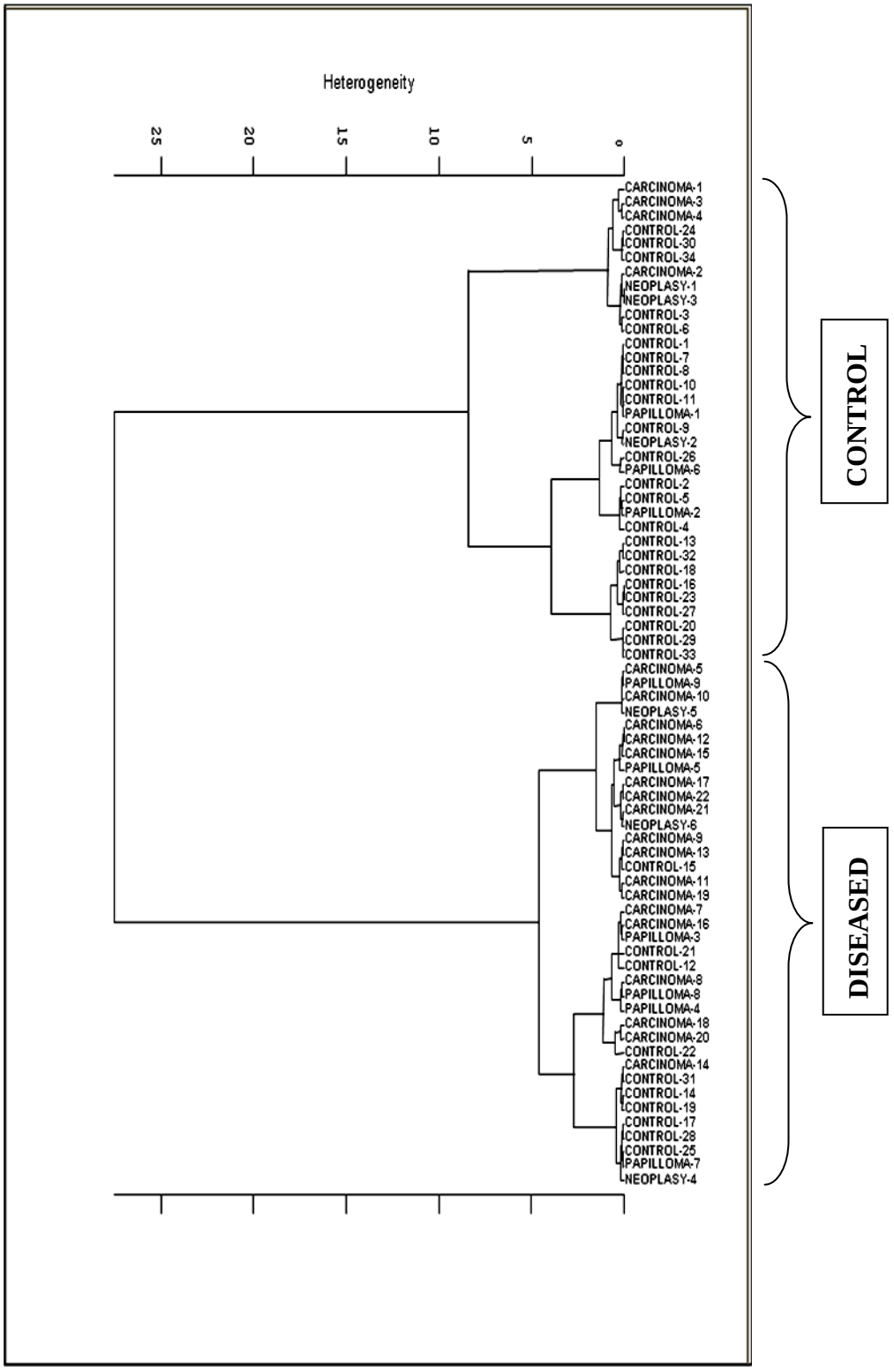


Figure 25: Hierarchial clustering of control and diseased samples using second derivative spectra (spectral range: 1015-1033 cm^{-1}). The sensitivity is 72.9% and the specifity is 70.6%.

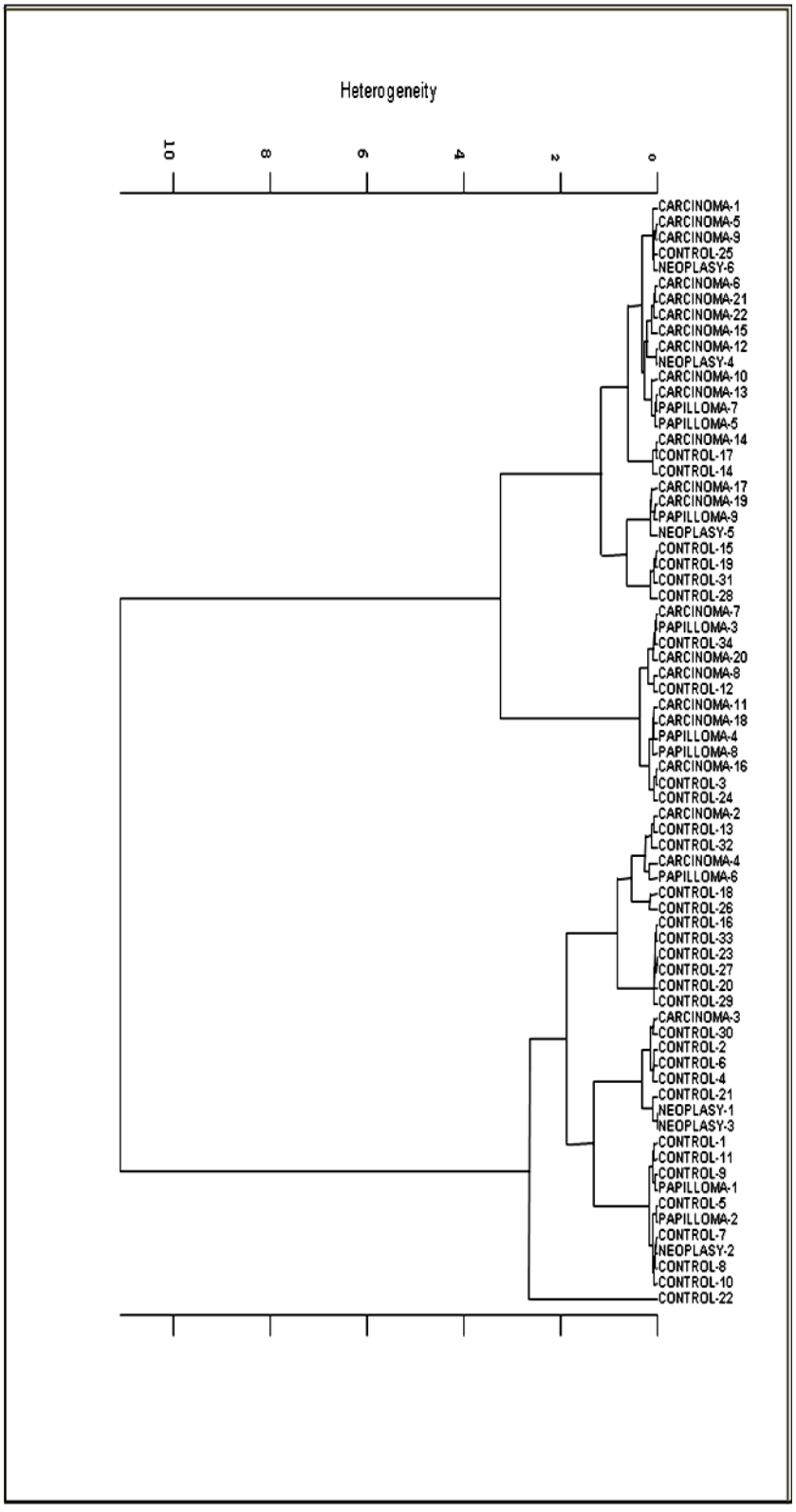


Figure 26: Hierarchial clustering of control and diseased samples using second derivative spectra (spectral range: 637-649cm^{-1}). The sensitivity is 75.7% and the specifity is 70.6%.

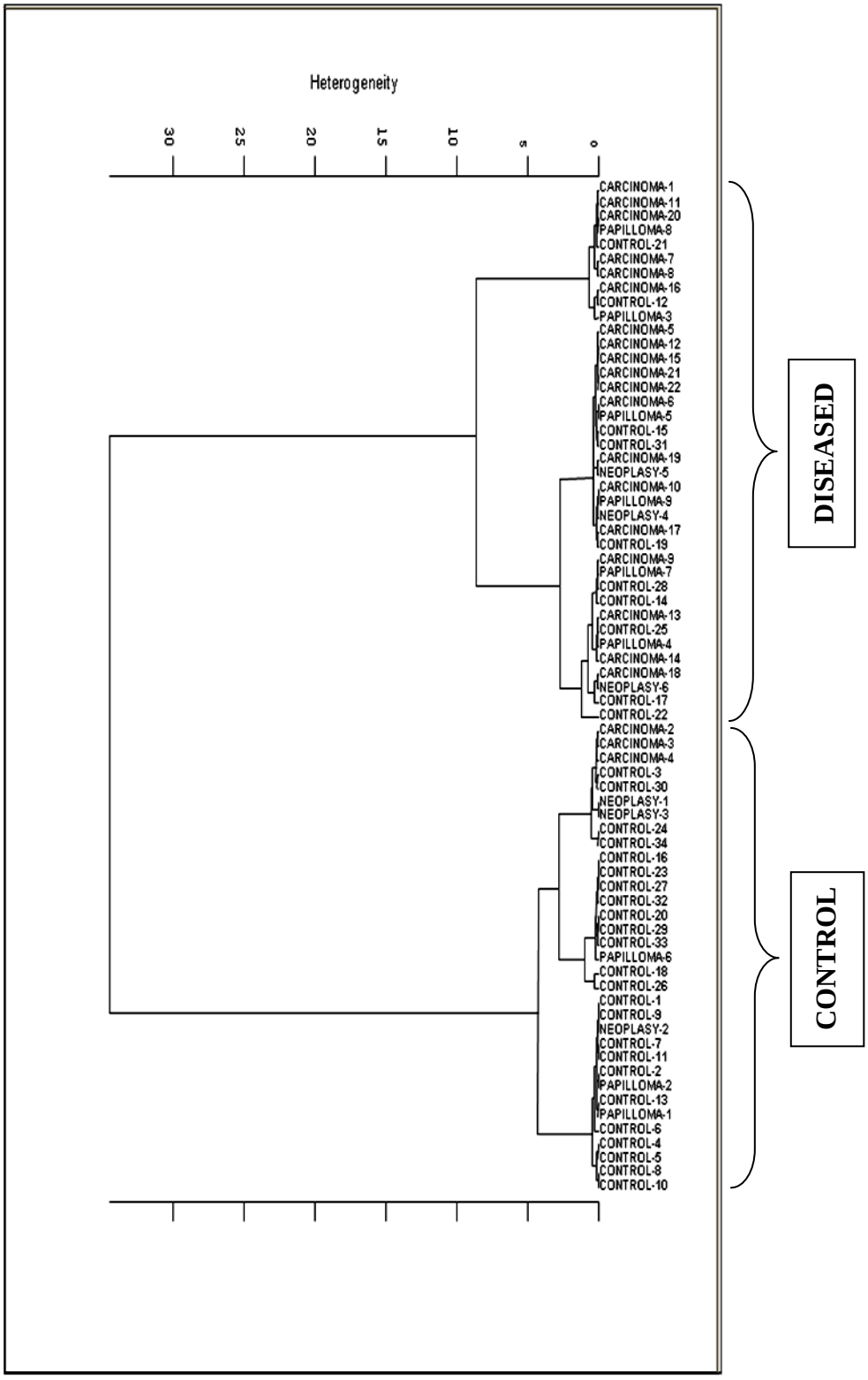


Figure 27: Hierarchial clustering of control and diseased samples using second derivative spectra (spectral range: 625-637cm^{-1}). The sensitivity is 75.7% and the specifity is 70.6%.

On the basis of the biopsy and cystoscopy results, the sensitivity and specifity of the spectral ranges were calculated. The results were seen in Table 12.

CHAPTER 4

DISCUSSION

The groups investigated in this study were chosen to represent the range of pathologic features commonly encountered by the practicing urologist. Although we could not classify the tumors because of low sample size of PUNLMP and papilloma groups, we could detect them. Therefore, FT-IR spectroscopy has been shown to be capable of detecting low stage tumors in the bladder.

In infrared spectroscopic analysis, the frequency of the bands gives information about the structure/conformation and intermolecular interactions, while the area under the bands monitors the concentration of the related molecules (Freifelder, 1982; Toyran *et al*., 2004).

The amide A band located at 3282 cm^{-1} in the control group, arises mainly from the N–H and the O–H stretching modes and intermolecular H bonding (Melin *et al.*, 2000; Cakmak *et al.*, 2006). In the present study, water was largely removed in the preparation of the bladder wash pellets. Therefore, the contribution of water molecules to this band could be neglected. There was a significant shift to lower values in the frequency of amide A in the carcinoma group compared to the control group. Many compounds contribute to this band. In our case, other than resectisol, different molecules contribute to amide A band such as urea and creatinine found in the urine and protein and polysaccharides found in the cells. Therefore this band was not taken into consideration.

The band at 2877 cm^{-1} is attributable to the CH_3 symmetric stretching band, which is mainly due to proteins and amino acids (Melin *et al.*, 2000; Cakmak *et al.*, 2003). This peak can be a good criterion to detect the tumor since the shift in the frequency of this band was significant in all tumor groups. The significant

shift in the frequency to lower values as the tumor appeared, suggested a change in the structure of the proteins. Moreover, the area of the band increased which suggested an increase in the protein content in carcinoma group. This result was not observed in the C=O stretching band located at 1664 cm^{-1} which is generally observed to detect the protein content. However since in our case, urea and creatinine also effect the C=O stretching band, this band was not also used to detect protein content. Instead, the CH_3 symmetric stretching band located at 2877 cm^{-1} in the control group, which is not effected by the urea and creatinine existence, was used to investigate the protein content. A possible reason for this result may be the appearance of some cancer specific proteins. It has been well known that cancerous cells cause overexpression of the glycoproteins levels, or formation of abnormal proteins and/or glycoproteins. Abnormal glycoproteins may mean abnormal sugar moieties (e.g. increased branching of N-linked glycans sugar chains) or misfolding because of the change in hydrogen bonding. These cancer spesific proteins and glycoproteins are used to detect the bladder cancer such as NMP22, C7orf24 proteins (Kageyama *et al.*, 2007). Glycoproteins overexpression was demonstrated in several malignancies (Hochhauser *et al.*, 1991) and is one mechanism by which cells acquire multidrug resistance (Gottesman *et al.*, 1996; Lum *et al.*, 1993). In tumor cells, these glycoproteins are thought to reduce intracellular drug concentrations, thereby limiting access of chemotherapeutic substrates to the site of action (Stein *et al.*, 1997). Because of this phenomenon, inhibition of some specific glycoproteins (such as P-gp) is an area of intense research as a potential method of restoring tumor cell sensitivity to chemotherapeutic agents.

This possible reason (overexpression of the glycoproteins, or formation of abnormal proteins and/or glycoproteins) is also supported by the significant increase in the area of the carbohydrate bands (band 8 and band 9) in all tumor samples. Same result was also shown before in some studies (Liu *et al.*, 2006). Another change in carbohydrate bands was the significant shift in the frequencies of C–O stretching and C–O bending bands (band 8 and band 9) to higher values in all tumor samples which can suggests a change in the

composition of the saccharides. This result is also consistent with the fact that the abnormal glycoproteins produced in cancerous cells. These glycoproteins carry abnormal sugar chains compared to normal cells. Most malignant cells have a common feature of increased branching of N-linked glycans (Orntoft *et al.*, 1999).

Cancer considerably induces several changes in the membrane properties. These changes take place in different ways in different parts of the membrane. The changes in the deep interior of the bilayer can be monitored by the wavenumber of the CH_3 antisymmetric stretching mode and an increase in the frequency of this band reflects increasing librational freedom of the acyl chains in the central area of the bilayer (Umemura *et al.*, 1980). The significant cancer-induced shift in the frequency of this band to higher values suggested that there was an increase in the librational freedom of the acyl chains of the phospholipids in the central area of the bilayer of tumor samples. In addition, the area of the CH_3 antisymmetric stretching mode significantly increased in carcinoma group which meant that the number of methyl groups in the acyl chains of lipids is higher in carcinoma group.

One result that was expected but not observed was the change in the dynamics of the system which can be monitored by the bandwidth of the CH_2 asymmetric stretching mode band located at 2936 cm^{-1}. Since the same amount of sample was used in all the experiments, any changes in the bandwidths of the CH_2 stretching modes reflect fluidity changes in membranes (Mantsch *et al.*, 1984). According to our results, there was not a considerable tumor-induced change in the dynamics of the membrane in diseased samples. The membrane fluidity has been studied in cancer for a long time and it seems that in most cases an increase of membrane fluidity accompanies the process of cell transformation (Nakazawa *et al.* 1989; Zirong *et al.*, 1991; Ben-Bassat *et al.*, 1977). Membrane fluidity of the tumor cells is thought to have an influence on metastasis. It has been found that domain organization and fluidity of the cell membrane affect tumor cell adhesion and in this way can have an impact on the malignancy of breast cancer

cells (Zeisig *et al.,* 2007). Ambrus *et al.* (1981) in the study on influence of phosphodiesterase inhibition on membrane fluidity and cell deformability proposed that cells with more fluid and flexible membranes may pass the microcirculation easier and are less likely to settle and form metastases.

On the other hand, the shift in the frequency of the CH_2 antisymmetric band in the carcinoma samples to lower wavenumbers correspond to an increase in number of *trans* conformers (Severcan, 1997). This result might imply that as the tumor appeared, the cells had more ordered membrane structure because it was composed of more *trans* conformers in the fatty acyl chains of membrane phospholipids. In other words, acyl chain flexibility decreased and they are more tightly packed. This increase in membrane order might be the result of the altered proportion of unsaturated acyl chains in triglycerides and cholesterol esters, as also suggested Nara *et al.* (2002). The membrane order also has been studied in cancer for a long time like membrane fluidity. However there are contradictory results in the literature relating to the change in membrane order in cancer. Supporting our studies, Galeotti *et al.* (1986) also observed increase in membrane order in cancer (hepatoma) cells and they suggest that, in the case of hepatomas a correlation exists between the growth rate of the tumor, the chemical composition of the membrane (in terms of lipid content, cholesterol content, and degree of fatty acid unsaturation), and the molecular order of lipid bilayer. On the other hand, Barker and Bowler (1991) showed that the membranes from cells of D23 hepatoma and MC7 sarcoma tumors are significantly less ordered than those of healthy liver.

The C=O stretching band located at 1664 cm^{-1} was widely used in previous cancer studies with FT-IR spectroscopy, since this band gives important information about the concentration and the conformational changes of proteins (Andrus *et al.,* 1998; Sahu *et al.,* 2004). Changes in protein concentration and/or conformation can give important clues about tumoral changes in tissues and can provide information about the cancer mechanism since it has been well known that cancerous cells causes abnormal proteins and/or glycoproteins formation.

However in our case, since urea exists in the sample and it has very strong contribution to the C=O stretching band, protein contribution to this band from the cells is neglected. There was a significant decrease in the area of this band in carcinoma group. We can state that there was a decrease in the urea concentration in carcinoma group. However urea concentration is affected by many factors such as the diet and kidney efficiency. So the decrease in urea concentration is not informative about tumoral changes. On the other hand, the significant shift in the frequency of the urea bands can be informative about tumoral changes. The significant shift in the frequency of the C=O stretching band (which is effected highly by the urea) to lower values in the carcinoma group (and papilloma group) may suggest a conformational change in urea. In another band with strong urea contribution (1462 cm^{-1}) also, there was a significant shift to higher values which again suggest a structural change in urea. Another possibility is a decrease in the urea hydrogen bonding. Urea is one of the simplest molecules in biology, but one of the most important. Hydrogen bonding is important in the solubility of the urea. In particular, the urea-water system has been extensively studied because of its significance in living beings. However, the behavior of urea in water is still not clearly understood. On the other hand, Hoccart and Turrell (1995) suggest that in Raman spectroscopy more hydrogen bonding of urea shifts the C-N stretching band to lower frequencies which is in accordance with our result.

The band which overlapped with the C-N antisymmetric stretching band (band 6) of the urea, was CH_2 bending mode of mainly lipids (Cakmak *et al.*, 2003; Manorahan *et al.*, 1993). A significant shift in the frequency of this band was also observed . The frequency of this band gives information about interchain packing and order/disorder properties of lipid bilayers. However to study the interchain packing and order/disorder properties of lipid bilayers, the pressure dependence of this mode has been widely used (Wong *et al.*, 1993; Wong *et al.*, 1990). Since the pressure dependence of this mode was not investigated in this study, the C-H stretching region is taken into consideration. The C-H stretching vibrations of the fatty acyl chains of all cellular lipids as well as protein groups

lead to bands in the 3050-2800 cm^{-1} spectral region. However the significant shift in all tumor types shows that this band can be a good criterion to detect the bladder tumor.

A considerable change was the increase in nucleic acid content and conformation in the case of tumor appearance and/or progression. This change has been well documented in the literature before (Wong *et al*, 1991; Wong *et al* 1993; Liu *et al.*, 2006). Since tumor is a situation in which a group of cells display uncontrolled growth and abnormal cell division, considerable alterations take place in the nucleic acid concentration and conformation. The increase in nucleic acid signals can arise because of the processes of transcription and DNA duplication during carcinogenesis in cancer cells/tissues compared to normal. Another possible reason is the increase in the nuclei-to-cytoplasm ratio which is commonly observed in cancer tissues (Sternberg *et al*, 1989). Other than the area increase, there was a significant shift to higher values in the frequencies of the peaks related to nucleic acids (band 10 and 11) in diseased samples. A decrease in the frequency implies either the strengthening of existing hydrogen bonding or formation of new hydrogen bonding between the components (Rigas *et al.*, 1990; Wong *et al.*, 1991). Hydrogen bonding has important role in DNA stability, and change in hydrogen bonding may affect the stability of DNA. Therefore our results suggest lower hydrogen bonding or weakening of hydrogen bonding in diseased samples which may mean a decrease in DNA stability.

There was a significant shift to higher values in the frequency of the PO_2^- symmetric stretching band (band 7) in carcinoma samples. In addition, the area of the symmetrical PO_2^- stretching band is significantly increased in the carcinoma and papilloma spectra as compared to the control spectra. Both the increased band area and shifted peak position to a higher frequency were commonly observed before in many different human cancers, including those of colon, esophagus, stomach, liver, ovary, cervix, basal cell carcinoma and these results indicate that in bladder tumors hydrogen bonding of the phosphodiester

groups of nucleic acids increases (Wong *et al.*, 1993; Rigas *et al.*, 1990; Wong *et al.*, 1990; Wong *et al.*, 1991).

Other than these bands, there are some other bands which can be used to detect tumoral changes, although they could not be assigned. However cancer induced significant changes in the frequency or area of these bands which were located at 2914 $cm^{-1,}$ 953 cm^{-1} and 629 cm^{-1} in the control group.

As seen from the figures, control and carcinoma samples show different FT-IR spectra. On the basis of these spectral differences, cluster analysis was performed using the bands that are significantly different between groups to differentiate between the control and carcinoma individuals spectra. We observed different regions to verify the results and to increase the success of the study. Hierarchial clustering of the control and carcinoma samples using second derivative spectra in the spectral ranges of 2907-2923 cm^{-1}, 1444-1457 cm^{-1}, 637-649 cm^{-1} and 625-637 cm^{-1} gave best results. We reached to a sensitivity of 100% sensitivity in spectral ranges 2907-2923 cm^{-1} and 1444-1457 cm^{-1}, while we obtained 86.4% sensitivity in spectral ranges 637-649 cm^{-1} and 625-637 cm^{-1} in the hierarchial clustering of control and carcinoma samples. These sensitivities are quite succesful compared to the sensitivity of urine cytology. In our study, urine cytology had a sensitivity of only 45% in carcinoma patients (Table 12).

Table 11: Sensitivities and specifities of the spectral ranges used for clustering of control and carcinoma samples

Spectral Ranges (cm^{-1})				
	2907-2923	**1444-1457**	**637-649**	**625-637**
Sensitivity (%)	**100**	**100**	**86.4**	**86.4**
Specifity (%)	**58.8**	**61.8**	**67.7**	**70.6**

Not only control and carcinoma samples, but also control and PUNLMP-papilloma samples (which are very difficult to detect with routine methods) show different FT-IR spectra. Therefore we also wanted to investigate the success of our study in low grade tumors (PUNLMP and papilloma) and we performed the hierarchial clustering of the samples. Hierarchial clustering of the control and diseased samples using second derivative spectra in the spectral ranges of 2954-2979 cm^{-1}, 2907-2923 cm^{-1}, 1444-1457 cm^{-1}, 1015-1033 cm^{-1}, 637-649 cm^{-1} and 625-637 cm^{-1} gave best results. In this case, we reached to an overall sensitivity (including all individuals with tumor) of 91.8% in spectral range 1444-1457 cm^{-1}, while we obtained an overall sensitivity of 83.8% in spectral range 2954-2979 cm^{-1} and 2907-2923 cm^{-1}, 75.7% in spectral ranges 637-639 cm^{-1} and 625-637 $cm^{-1,}$ and 72.9% in spectral region 1015-1033 cm^{-1}. We did not perform the clustering of control and PUNLMP or control and papilloma samples, since the sample size in PUNLMP and papilloma groups were insufficient. However, we observed that especially in the spectral ranges of 1444-1457 cm^{-1}, 2954-2979 cm^{-1} and 2907-2923 cm^{-1} the sensitivities of our method for papilloma group were 77.8%, 66.7% and 66.7% , respectively. In our study, urine cytology had a sensitivity of only 11.1% (Table 12). The sensitivity for PUNLMP group was 83.3% in these 3 spectral ranges while the urine cytology had a sensitivity of only 16.7% (Table 12). Therefore the sensitivity of our method in overall and per grade, was much higher than cytology.

Table 12: Sensitivities and specifities of the spectral ranges used for clustering of control-diseased samples and cytology in our study

	Sensitivity (%)	Carcinoma Sensitivity (%)	PUNLMP Sensitivity (%)	Papilloma Sensitivity (%)	Specifity (%)
2954-2979	**83.8**	**90.9**	**83.3**	**66.7**	**67.7**
2907-2923	**83.8**	**90.9**	**83.3**	**66.7**	**67.7**
1444-1457	**91.8**	**100.0**	**83.3**	**77.8**	**61.7**
1015-1033	**72.9**	**81.8**	**50.0**	**66.7**	**70.6**
637-639	**75.7**	**86.4**	**50.0**	**66.7**	**70.6**
625-637	**75.7**	**86.4**	**50.0**	**66.7**	**70.6**
CYTOLOGY	**32.4**	**45.5**	**16.7**	**11.1**	**100.0**

Table 13: Overall sensitivities and specifities of the commonly used techniques for bladder tumors (Van Rhijn *et al*., 2009).

	SENSITIVITY (%)	SPECIFITY (%)
BTA	**58**	**73**
NMP ELISA	**69**	**73**
NMP POC	**62**	**86**
uCYT	**77**	**74**
UROVISION	**65**	**83**
MICROSATELLITE	**73**	**76**
CYTOLOGY	**35**	**94**

Other than the sensitivity advantage of our method over cytology, our method gives the results in much shorter time and it is much more cost-effective compared to cytology. In addition, the results were not dependent on the subjective observation of a pathologist, but to computational analysis of the spectra. In other words it is operator independent which is a very important advantege of our method. Furthermore, the FTIR spectra may give information

about the tumoral changes long before they become visible. So this information may be very important in early diagnosis. FTIR spectroscopy has been previously applied to determine early diabetes induced alterations (Toyran *et al.*, 2006).

We compared our overall sensitivity results to the overall sensitivities of other techniques namely BTA, NMP Elisa, NMP POC, uCYT, urovision, microsatellite and cytology (not in our study). These techniques are well known examples of urine based and cell based techniques Van Rhijn *et al.*2009. Although they have higher specifity than ours, our sensitivity was higher than the sensitivities of all these techniques. Among these techniques, most sensitive one was uCYT with 77% sensitivity and 74% specifity (Table 13) and yet it could not reach to the sensitivity (91.8%) of our most sensitive spectral range (1444-1457 cm^{-1}). We believe that by increasing patient number, our specifity will also increase.

The patient number in our study was insufficient to classify the tumors, but FT-IR has been shown to be capable of detecting tumors with good accuracy levels in the bladder. A difficulty that we encountered was to find control patients who were healthy for sure, since the cystoscopy is not applied to persons unless there is a suspicion for bladder tumor. However, when the pathologist does not find tumorous change in the biopsy or cytology, this does not mean that this person is totally healthy, there is a possibility that this person will develop bladder tumor in the future. In two individuals who were diagnosed as healthy/without tumor by the pathologist and who were consistently clustered in diseased group in our study, developed bladder tumor in a year. This may mean that FT-IR spectroscopy detects very small changes in the bladder wash samples that the pathologist can not observe. By monitoring other samples in our study which conflict with the pathology results in the following months, we should be certain about the truth of differentiation. If this is indeed true, FTIR spectroscopy will provide a very early detection of the tumor which may prevent the high grade tumor development and lower the expenses required to destroy the tumor.

CHAPTER 5

CONCLUSION

Bladder tumor involves a long-term therapy period which requires frequent controls and this causes the cost to rise. In addition, since cystoscopy performed during follow-up disturbs the comfort of the patient, this sometimes leads the patient to discontinue to follow-up. Another problem is that the biopsy interpretation can be highly subjective and there are inconsistencies between the results of different pathologists and even between the different results of the same pathologist. Also, interpretations of biopsies can be confounded by sampling problems such as the absence of the muscular layer in the specimen, or the exclusion of the bladder wall in biopsies of large tumours growing exophytically that can affect the staging. Most significantly, the basic tools available to determine tumour behaviour, malignant potential and chance for recurrence provided by the current pathological staging methods can be highly subjective. A new approach is needed which is automated, which is not costly, and which does not disturb patient comfort. For this purpose, we used FT-IR spectroscopy in this study on the bladder wash sample collected during cystoscopy. This study is unique since it is the first study that uses the bladder wash sample -the content of which is close to the urine- in the diagnosis of the bladder tumor.

Our results showed that FT-IR can be used to detect the bladder tumors in bladder wash sample. FT-IR gave results in much shorter time and is much less costly than cytopathologic methods. In addition, for the first time we showed the efficiency of the FT-IR spectroscopy to the urine cytology which is golden standard. It detected the low stage tumors with higher sensitivity compared to cytology. In the long term, with sufficient sample size, FT-IR would be able not only detect but also to classify the tumors. Another step after our study can be to

use urine instead of bladder wash sample (the contents of both are nearly same) which will not disturb patient's comfort and increase the patient's concordance.

In summary, we propose the utilization of the FT-IR technique for the detection of bladder tumors since specific wavenumbers have shown to be effective markers. In addition, the utilization of FT-IR which can shed some light on the possible pathway or disease progression.

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