A NEW APPROACH IN THE SCREENING FOR CERVICAL CANCER USING FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

DR. SHADY GHALEB MAHMOUD EL-TAWIL

UNIVERSITI SAINS MALAYSIA
2008
Light upon light! God doth guide whom He will to His light: God doth set forth parables for men: and God doth know all things [Al Nur, 35]
DEDICATION

To my beloved parents, may ALLAH Al-mighty save them
ACKNOWLEDGEMENTS

Praise and thanks to ALLAH, the Lord, AL-Mighty for giving me the strength and patience throughout the duration of my study.

First and foremost I would like to thank and extend my grateful appreciation to my main supervisor, Professor Dr. Nor Hayati Othman who had led me with her persistent motivation, financial and emotional support and farsighted guidance from the beginning of the proposal writing until the submission of this dissertation, I am indebted to her for her endless help.

My special thanks also to my co-supervisors Dr. Rohana Adnan and Associate Professor Dr. Nik Muhamed Zaki for their expert advice throughout my research work. Also a lot of thanks to Dr. Mohd Ayub Sadiq for his expert analytical and mathematical contributions to this study.

I also would like to thank Universiti Sains Malaysia for providing short term grant (304/PPSP/6131442) to cover the cost of this study.

My respect and thanks to all the staff at Pathology Research Laboratory and at School of chemical Sciences for their continuous help.

I am very grateful to have the assistance of Mr. Selvarajan for his assistance in providing the samples from Gribbles Pathology Laboratory during the study, and
Mr. Aw Yeong from school of chemical sciences for providing the skilled training to use FTIR spectroscopy.

My sincere and special gratitude to Associate Professor Dr. Nik Zainal Abidin Nik Ismail from Pediatrics department for his help by giving me the chance to be in Malaysia and pursue my postgraduate study in Universiti Sains Malaysia, also my deepest thanks to Professor Dr. Aziz Baba for his great advices and assistance and to Professor Zainul Fadziruddin Zainuddin and Associate Professor Dr. Syed walliullah for allowing us to use FTIR spectroscopy in school of Health Sciences.

I also extend my thanks to my friends for their support, especially Zulkefli Sanip and Nordyiah bnt Othman, may our relationship last forever and ever.

I would also not forget the continuous and unlimited support given by my loving parents and Enemesia El-tawil my beloved wife for providing their never ending love, support and tolerance to enable me to finish this dissertation and complete my Master of Science degree in Pathology.

I also should not forget my brothers Mohamed, Ahmed and my sister Shaimaa for giving extra support for me to finish my study.

To all named and unnamed friends and helpers, I again extend my thanks.

Dr. Shady G. El-tawil
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xviii</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xx</td>
</tr>
<tr>
<td>LIST OF PUBLICATIONS</td>
<td>xx</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>xxi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xxiv</td>
</tr>
<tr>
<td>CHAPTER ONE : INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Statement of the current problem</td>
<td>5</td>
</tr>
<tr>
<td>CHAPTER TWO : OBJECTIVES</td>
<td>6</td>
</tr>
<tr>
<td>2.1 General objectives</td>
<td>7</td>
</tr>
<tr>
<td>2.2 Specific objectives</td>
<td>7</td>
</tr>
<tr>
<td>2.3 Hypothesis</td>
<td>7</td>
</tr>
</tbody>
</table>
CHAPTER THREE: LITERATURE REVIEW

3.1 Cervical cancer

3.1.1 Background

3.1.2 Burden of suffering

3.1.3 Etiology
   3.1.3 (a) Human Papilloma Virus (HPV)
   3.1.3 (b) Sexually transmitted diseases (STD)
   3.1.3 (c) Nutritional factors
   3.1.3 (d) Oral contraceptives and IUDs
   3.1.3 (e) Genetic aspects
   3.1.3 (f) Other risk factors

3.1.4 Epidemiologic studies

3.1.5 Cancer of the cervix in Malaysia

3.1.6 Pap smear tests

3.1.7 Pap smear interpretation
   3.1.7(a) The normal squamous cells
   3.1.7(b) Low grade squamous intraepithelial lesion (LSIL)
   3.1.7(c) High grade squamous intraepithelial lesion (HSIL)
   3.1.7(d) Squamous cell carcinoma (SCC)

3.1.8 Screening of cervical cancer
   3.1.8(a) Initiation and frequency of screening
   3.1.8(b) Recommendations to stop screening
   3.1.8(c) Reporting screening results

3.1.9 Screening failures
3.1.10 Diagnosis of cervical cancer 46

3.1.11 Management of women based on pap smear results 48

3.1.12 Treating precancerous cervical lesions 52
   3.1.12(a) Cryotherapy 53
   3.1.12(b) Loop Electrosurgical Excision procedure (LEEP) 55
   3.1.12(c) Conization of cervix 56

3.1.13 Staging of cervical cancer 56

3.1.14 Treatment of cervical cancer 58

3.2 Fourier Transform Infrared Spectroscopy (FTIR) 63
   3.2.1 Introduction 63
   3.2.2 The electromagnetic spectrum 65
   3.2.3 Electromagnetic interaction with matter 67
      3.2.3(a) Absorbance 67
      3.2.3(b) Scattering 68
      3.2.3(c) Emission 68
   3.2.4 Theory of infrared absorption 69
   3.2.5 How it works 71
   3.2.6 Dispersive spectrometers 72
   3.2.7 Fourier transform spectrometers 72
   3.2.8 Spectrometer components 73
3.2.9 Spectrometer design 78

3.2.10 FTIR spectroscopy advantages 79

3.2.11 Analytical uses of FTIR spectroscopy 81

3.2.11(a) Qualitative analysis 81
1. Structural elucidation 82
2. Compound identification 86

3.2.11(b) Quantitative analysis 87

3.2.12 Calibration methods 88

3.2.13 Theory of fitting procedure 89

3.2.14 Method of partial least squares (PLS) 90

3.2.15 Application of FTIR spectroscopy to medical research 91

3.2.16 Limitations of FTIR spectroscopy 93

CHAPTER FOUR: MATERIALS AND METHODS 95

4.1 Study design 96

4.2 Time of study 96

4.3 Study subjects 97

4.3.1 Inclusion criteria 97
4.3.2 Exclusion criteria 97
4.3.3 Sample size calculation 97
4.3.4 Approval for study 98
4.4 Collection of the samples

4.5 Materials used for cytological examination

4.6 Preparation of samples for cytopathology

4.7 FTIR spectroscopy analysis
   4.7.1 Material used
   4.7.2 Optimizing FTIR analysis
   4.7.3 Identification of reference (standard) spectra
   4.7.4 Spectral preprocessing
   4.7.5 Spectroscopic identification of unknown samples
   4.7.6 The standard FTIR spectroscopy parameters

4.8 Statistical analysis

4.9 Flow chart of the study

CHAPTER FIVE: RESULTS

5.1 Demographic results
   5.1.1 Age
   5.1.2 Ethnicity

5.2 Cytological results

5.3 FTIR analysis results
   5.3.1 Results of Calibration
   5.3.2 FTIR spectra of the reference samples
5.3.2(a) Spectrum for normal cells 129
5.3.2(b) Spectrum for cells with LSIL 130
5.3.2(c) Spectrum for cells with HSIL 131
5.3.2(d) Spectrum for cells with Cancer 132

5.3.3 The peak values of chemical components of the reference samples 133

5.3.3(a) The peak value of glycogen 133
5.3.3(b) The peak value of symmetric phosphate in nucleic acids 134
5.3.3(c) The peak value of carbonyl in carbohydrates 135
5.3.3(d) The peak value of asymmetric phosphate in nucleic acids 136
5.3.3(e) The peak value of carbonyl in proteins 137
5.3.3(f) The peak value of amide-2 in proteins 138
5.3.3(g) The peak value of amide-1 in proteins 139

5.3.4 The ratio values of different chemical components 140

5.3.4(a) Glycogen/ nucleic acid ratio 140
5.3.4(b) Nucleic acid-2/ carbohydrates ratio 141
5.3.4(c) Carbohydrates/ protein ratio 142
5.3.4(d) Nucleic acid-1/ amide-2 ratio 143
5.3.4(e) Protein/ amide-1 ratio 144
5.3.4(f) Protein/ nucleic acid-1 ratio 145

5.3.5 Peak locations 146
5.3.6 Band intensity ratios 147
5.3.7 Test of significance 148

5.4 Comparative results between cytology and FTIR method 150

5.4.1 Measurement of agreement between cytology and FTIR method 151
5.4.2 The accuracy of FTIR spectroscopy 152
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Cervix uteri cancer incidence per 100,000 population (CR) and Age-standardized incidence (ASR), Peninsular Malaysia 2003</td>
<td>23</td>
</tr>
<tr>
<td>3.2</td>
<td>Cervix uteri cancer incidence per 100,000 population (CR) and Age-standardized incidence (ASR), by ethnicity, Peninsular Malaysia 2003</td>
<td>25</td>
</tr>
<tr>
<td>3.3</td>
<td>WHO histological classification of tumours of uterine cervix</td>
<td>37</td>
</tr>
<tr>
<td>3.4</td>
<td>Guidelines for the management of women based on Pap smear results</td>
<td>50</td>
</tr>
<tr>
<td>3.5</td>
<td>Recommendations of other groups about Pap smear for cervical cancer screening</td>
<td>51</td>
</tr>
<tr>
<td>3.6</td>
<td>International Federation of Gynecology and Obstetrics (FIGO) surgical staging systems for carcinoma of the uterine cervix</td>
<td>57</td>
</tr>
<tr>
<td>3.7</td>
<td>Chemotherapy regimens for recurrent or metastatic cervical cancer</td>
<td>62</td>
</tr>
<tr>
<td>3.8</td>
<td>Correlation chart with a number of functional groups in IR region between 4000 - 400 cm(^{-1})</td>
<td>85</td>
</tr>
<tr>
<td>4.1</td>
<td>The experimental condition for FTIR analysis in our study</td>
<td>120</td>
</tr>
<tr>
<td>5.1</td>
<td>Average peak frequencies (means ± SD) of chemical components in normal, LSIL, HSIL and cancer cells spectra</td>
<td>146</td>
</tr>
<tr>
<td>5.2</td>
<td>Absorbance intensity ratios of biochemical components of normal, LSIL, HSIL and cancer cells spectra using FTIR spectroscopy (means ± SD)</td>
<td>147</td>
</tr>
<tr>
<td>5.3</td>
<td>Non-parametric (Kruskal Wallis) test of significance between the peak frequencies of the four groups ((P&lt;0.05))</td>
<td>148</td>
</tr>
<tr>
<td>5.4</td>
<td>Analysis of variance (ANOVA) between the absorbance intensity ratio of the four categories ((P&lt;0.05))</td>
<td>149</td>
</tr>
<tr>
<td>5.5</td>
<td>Results of both ThinPrep (gold standard) test and FTIR spectroscopy</td>
<td>150</td>
</tr>
<tr>
<td>5.6</td>
<td>Measurement of agreement using kappa test</td>
<td>151</td>
</tr>
</tbody>
</table>
5.7 Comparison between the results of the Thin Prep cytology with FTIR spectroscopy (Normal vs LSIL, HSIL & Cancer)  
5.8 Sensitivity and specificity of FTIR spectroscopy to detect LSIL, HSIL and SCC as abnormal results  
5.9 Comparison between the results of the Thin Prep cytology with FTIR spectroscopy (Normal & LSIL vs HSIL & Cancer)  
5.10 Sensitivity and specificity of FTIR spectroscopy to detect abnormal results (HSIL and SCC only)
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Age-standardized incidence rates of cervical cancer in developed and developing countries for the year 2005</td>
<td>10</td>
</tr>
<tr>
<td>3.2</td>
<td>Age-standardized mortality rates of cervical cancer in developed and developing countries for the year 2005</td>
<td>11</td>
</tr>
<tr>
<td>3.3</td>
<td>Diagram represents a current understanding of cervical cancer natural history</td>
<td>20</td>
</tr>
<tr>
<td>3.4</td>
<td>Ten most frequent cancers in females, Peninsular Malaysia 2003</td>
<td>23</td>
</tr>
<tr>
<td>3.5</td>
<td>Cervix uteri Age- specific cancer incidence per 100,000 population, Peninsular Malaysia 2003</td>
<td>24</td>
</tr>
<tr>
<td>3.6</td>
<td>The prevalence of cancer of the cervix in Malaysia 1985-2001</td>
<td>27</td>
</tr>
<tr>
<td>3.7</td>
<td>Superficial (small arrow) and intermediate (large arrow) squamous cells with normal polygonal epithelial cells</td>
<td>30</td>
</tr>
<tr>
<td>3.8</td>
<td>Low grade squamous intraepithelial lesion (LSIL)</td>
<td>33</td>
</tr>
<tr>
<td>3.9</td>
<td>High grade intraepithelial lesion (HSIL)</td>
<td>34</td>
</tr>
<tr>
<td>3.10</td>
<td>Invasive squamous cell carcinoma (SCC)</td>
<td>36</td>
</tr>
<tr>
<td>3.11</td>
<td>A graphical representation of the electromagnetic spectrum according to its wavelength</td>
<td>65</td>
</tr>
<tr>
<td>3.12</td>
<td>Major vibrational modes for nonlinear group of CH₂</td>
<td>71</td>
</tr>
<tr>
<td>3.13</td>
<td>Perkin-Elmer model FTIR spectroscopy</td>
<td>73</td>
</tr>
<tr>
<td>3.14</td>
<td>Simplified optical layout of a typical FTIR spectroscopy</td>
<td>75</td>
</tr>
<tr>
<td>3.15</td>
<td>Internal components of FTIR spectroscopy</td>
<td>79</td>
</tr>
<tr>
<td>3.16</td>
<td>Structure of polystyrene</td>
<td>89</td>
</tr>
<tr>
<td>4.1</td>
<td>Thin prep medium bottle and cytobrush</td>
<td>100</td>
</tr>
<tr>
<td>4.2</td>
<td>ThinPrep instrument</td>
<td>100</td>
</tr>
<tr>
<td>4.3</td>
<td>Thin Prep method steps</td>
<td>102</td>
</tr>
<tr>
<td>4.4</td>
<td>Barium fluoride window</td>
<td>103</td>
</tr>
<tr>
<td>4.5</td>
<td>Microspatula</td>
<td>103</td>
</tr>
</tbody>
</table>
4.6  Vacuum desiccator  104
4.7  Dehumidifier  104
4.8  FTIR screen displaying the parameters used to take background spectrum  107
4.9  FTIR spectrum of the background displaying the transmittance bands from H₂O and CO₂  108
4.10  FTIR screen displaying transmittance spectrum of both the background (black spectrum) and the known sample (blue spectrum)  109
4.11  The FTIR spectrum of the reference sample  110
4.12  FTIR screen displaying setup menu and compare order  112
4.13  FTIR screen displaying window of compare setup  113
4.14  FTIR screen displaying window of the reference spectra directory  114
4.15  FTIR screen with window of the range for comparison between spectra  115
4.16  FTIR screen displaying the process menu and compare order  116
4.17  FTIR screen displaying the progress in identification of the spectrum  117
4.18  FTIR screen displaying both the unknown IR spectrum (in black) and the reference IR spectrum (in blue)  118
4.19  FTIR screen displaying the result of identification of the unknown sample showing normal result  119
5.1  Histogram of the age frequency of the women involved in the study  124
5.2  Pie chart of ethnic distribution of subjects in this study  125
5.3  Pie chart of the percentage of the cytological results of collected Pap smear samples  126
5.4  FTIR spectrum of Polystyrene film  127
5.5  Infrared spectrum of Normal, LSIL, HSIL and Cancer cervical cells  128
5.6  Infrared spectrum of normal cervical smear  129
5.7  Infrared spectrum of LSIL cervical smear  130
5.8  Infrared spectrum of HSIL cervical smear  131
5.9 Infrared spectrum of SCC cervical smear

5.10 FTIR frequency of glycogen in the normal, LSIL, HSIL and SCC cervical smears

5.11 FTIR frequency of symmetric phosphate stretching in the nucleic acids of the normal, LSIL, HSIL and SCC cervical smears

5.12 FTIR frequency of carbonyl (C-O) stretching in the carbohydrates of the normal, LSIL, HSIL and SCC cervical smears

5.13 FTIR frequency of asymmetric phosphate (PO₃) stretching in nucleic acids of the normal, LSIL, HSIL and SCC cervical smears

5.14 FTIR frequency of carbonyl (C-O) in proteins of the normal, LSIL, HSIL and SCC cervical smears

5.15 FTIR frequency of amide-2 in the normal, LSIL, HSIL and SCC cervical smears

5.16 FTIR frequency of amide-1 in the normal, LSIL, HSIL and SCC cervical smears

5.17 Intensity ratio of glycogen/nucleic acid ratio in the normal, LSIL, HSIL and SCC cervical smears

5.18 Intensity ratio of nucleic acid-2/ carbohydrates ratio in the normal, LSIL, HSIL and SCC cervical smears

5.19 Intensity ratio of carbohydrates/ protein ratio in the normal, LSIL, HSIL and SCC cervical smears

5.20 Intensity ratio of nucleic acid/ amide-2 ratio in the normal, LSIL, HSIL and SCC cervical smears

5.21 Intensity ratio of protein/amide-1 ratio in the normal, LSIL, HSIL and SCC cervical smears

5.22 Intensity ratio of protein/ nucleic acid ratio in the normal, LSIL, HSIL and SCC cervical smears
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ANNs</td>
<td>Artificial neural networks</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ASCUS</td>
<td>Atypical squamous cell of undetermined significance</td>
</tr>
<tr>
<td>BaF₂</td>
<td>Barium fluoride</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTGS</td>
<td>Deuterated Triglycine Sulphate</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>HeNe</td>
<td>Helium Neon laser</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>HSIL</td>
<td>High grade squamous intra-epithelial lesion</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IUD</td>
<td>Intrauterine contraceptive device</td>
</tr>
<tr>
<td>KBr</td>
<td>Potassium bromide</td>
</tr>
<tr>
<td>LBC</td>
<td>Liquid based cytology</td>
</tr>
<tr>
<td>LSIL</td>
<td>Low grade squamous intra-epithelial lesion</td>
</tr>
<tr>
<td>MCT</td>
<td>Mercury Cadmium Telluride (HgCdTe)</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least squares</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for social sciences</td>
</tr>
<tr>
<td>STD</td>
<td>Sexually transmitted diseases</td>
</tr>
<tr>
<td>T</td>
<td>Transmittance</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZPD</td>
<td>Zero Path Difference</td>
</tr>
</tbody>
</table>
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Information and consent form (English)</td>
<td>188</td>
</tr>
<tr>
<td>1.2</td>
<td>Maklumat dan borang persetujuan (Malay)</td>
<td>193</td>
</tr>
<tr>
<td>1.3</td>
<td>Approval of the study by Research &amp; Ethics committee</td>
<td>198</td>
</tr>
<tr>
<td>1.4</td>
<td>Comparative results between cytology and FTIR spectroscopy</td>
<td>199</td>
</tr>
<tr>
<td>1.5</td>
<td>Steps of our methodology</td>
<td>210</td>
</tr>
<tr>
<td>1.6</td>
<td>Abstract submitted in MJMS, Malaysia, 2006</td>
<td>216</td>
</tr>
<tr>
<td>1.7</td>
<td>Abstract submitted in Modern Pathology, Canada, 2006</td>
<td>217</td>
</tr>
<tr>
<td>1.8</td>
<td>Full paper submitted in Pathology Journal, October, 2008</td>
<td>218</td>
</tr>
</tbody>
</table>
PENDEKATAN BARU DALAM PENYARINGAN KANSER SERVIK MENGGUNAKAN SPEKTROSKOPI FOURIER TRANSFORM INFRAMERAH

ABSTRAK

PENGENALAN:
Pap smear telah digunakan untuk mengesan petanda awal dan kanser serviks sejak 1940-an dan terdapat bukti yang jelas bahawa ujian Pap telah menurunkan kadar mortaliti/kematian disebabkan kanser serviks. Malangnya, terdapat keputusan negatif palsu yang mampu mencapai sehingga 69%. Spektroskopi FTIR digunakan dengan meluas dalam pelbagai bidang industri untuk menyukat dan mengesan kompound kimia. Terbaru, ia digunakan untuk mengkaji perubahan struktur pelbagai sel kanser manusia pada peringkat molekular.

OBJEKTIF:
Kajian ini dijalankan untuk menilai spektroskopi FTIR sebagai kaedah baru untuk penyaringan kanser serviks sebagai perbandingan dengan ujian sitologi.

METODOLOGI:
Serpihan serviks diambil dan ditempatkan di dalam medium ThinPrep™. Untuk analisis spektroskopi FTIR, sampel dikeringkan dan dipancarkan dengan sinaran infra merah pada frekuensi dari 4000 sehingga 400 cm⁻¹. Data serapan dicerap menggunakan spektrometer Spektrum BX II FTIR dan dibandingkan dengan bacaan serapan rujukan sampel yang diketahui menggunakan perisian spektroskopi FTIR. Bacaan spektroskopi FTIR dibandingkan dengan keputusan ujian sitologi.
KEPUTUSAN:

Spektroskopi FTIR dapat membezakan sel normal daripada sel abnormal pada 800 sampel yang dikaji. Spektrum normal dicirikan dengan jalur glikogen yang jelas/kuat pada 1,029 (±4.5) cm⁻¹, jalur meregang C-O karbohidrat yang agak jelas/kuat pada 1,156.4 (±4.7) cm⁻¹ dan jalur asimetrik fosfodiester asid nukleik pada 1,234 (±4.60) cm⁻¹.

Spektra abnormal dalam kecerunan berbeza menunjukkan penurunan pada intensiti jalur glikogen pada 1,029 (±4.5) cm⁻¹ dan intensiti jalur fosfodiester yang lebih jelas/kuat pada 1,234 (±4.6) cm⁻¹. Spektra abnormal juga dilihat apabila terdapat anjakan jalur simetri fosfodiester pada 1,078 (±1.6) cm⁻¹ kepada frekuensi yang lebih tinggi dan penurunan intensiti jalur C-OH pada 1,156 (±4.7) cm⁻¹. Sensitiviti untuk mengesan sel serviks abnormal adalah 85%, spesifisiti 91%, nilai jangkaan positif 19.5% dan nilai jangkaan negatif 99.5%.

KESIMPULAN:

Melalui kajian ini, kami mendapati bahawa FTIR mungkin berguna untuk penyaringan kanser serviks. Teknik ini mudah, pantas dan murah serta tidak memerlukan pengawetan dan pewarnaan. Kaedah ini dapat diaplikasi di negara yang tidak mempunyai sitopatologis yang mencukupi. Sepanjang pengetahuan kami, ini merupakan percubaan pertama menggunakan spektroskopi FTIR sebagai kaedah alternatif dalam penyaringan kanser serviks di Malaysia.
A NEW APPROACH IN THE SCREENING FOR CERVICAL CANCER USING
FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

ABSTRACT

INTRODUCTION:

Pap smear has been used successfully to detect precursor and cancer lesions of the uterine cervix since 1940s. There is clear evidence that Pap test has reduced mortality from cervical cancer. Unfortunately false negative smears occur and may reach up to 69%. Fourier transform infrared (FTIR) spectroscopy has been utilized to measure and detect chemical compounds in many industrial fields. Recently it has been used to study the structural changes of cells at the molecular level on various human cancers.

OBJECTIVES:

This study is to evaluate FTIR spectroscopy as new tool for screening of cervical cancer in comparison with cervical cytology.

METHODOLOGY:

Cervical scrapings were taken by cytobrush and placed in ThinPrep™ medium. The samples were dried over infrared transparent matrix. Beam of infrared light were directed at the dried samples at frequency from 4000 to 400 cm⁻¹. The absorption data were produced using Spectrum BX II FTIR spectrometer. Data were compared with the reference absorption data of known samples using FTIR spectroscopy software. FTIR spectroscopy was compared with cytology (gold standard).
RESULTS:

FTIR spectroscopy could differentiate normal from abnormal cervical cells in the 800 samples examined. The normal spectrum was characterized by strong glycogen band at 1,029 (±4.5) cm⁻¹, relatively strong C-O stretching band of carbohydrates at 1,156.4 (±4.7) cm⁻¹ and relatively weak band of the asymmetric phosphodiester stretching modes of nucleic acids at 1,234 (±4.6) cm⁻¹.

The abnormal spectra in different gradients showed a decrease in glycogen band intensity at 1,029 (±4.5) cm⁻¹, a stronger and broader intensity of phosphodiester stretching band at 1,234 (±4.6) cm⁻¹. Abnormal spectra were also seen when there was a shifting of symmetric phosphodiester band at 1,078 (±1.6) cm⁻¹ to a higher frequency and a decrease in the intensity of C-OH stretching band at 1,156 (±4.7) cm⁻¹. The sensitivity to detect abnormal cervical cells was 85%, specificity 91%, positive predictive value 19.5% and negative predictive value of 99.5%.

CONCLUSION:

With this preliminary study we conclude that FTIR may be a useful tool for screening of cervical cancer. The technique is simple, rapid and cheap. The cervical scrapings require no fixation or staining. This method could be adopted in countries where sufficient number of cytopathologists is not available. To the best of our knowledge this is the first attempt at using an alternative method for screening of cervical cancer using FTIR spectroscopy in Malaysia.
CHAPTER ONE

INTRODUCTION
INTRODUCTION

This work deals with a new screening test, Fourier Transform Infrared Spectroscopy (FTIR), an important and new technique for detection of cervical cancer at its early stages. Screening for cervical cancer is currently carried out by the Papanicolau (PAP) smear test. Squamous and glandular epithelial cells of transformation zone are exfoliated using Cytobrush or spatula, fixed in ethanol and stained with the Papanicolau stain (Patten, 1978).

Until the early 1990s, cervical cancer was the most frequent neoplastic disease among women in developing countries, before breast cancer became the predominant cancer. Each year, over 400,000 new cases of invasive cervical cancer are diagnosed world wide, representing nearly 10% of all cancers in women and was responsible for over 250,000 deaths in 2005 (WHO, 2006).

A definitive diagnosis is obtained by cervical biopsy and examination of the stained tissue. The predictive value of a biopsy is higher than that of the Pap test because the anatomical arrangement is preserved allowing evaluation of pathological features in relation to histological architecture (Yeoh and Chan, 1997).
Cervical disease is classified cytologically using the two-tier Bethesda system for Pap smears low- (LSIL) and high-grade squamous intraepithelial lesions (HSIL) and the three-tier cervical intraepithelial neoplasia (CIN I, II and III) system for surgical samples (LSIL=CIN I, HSIL= CIN II and CIN III) (Cestero, 2006).

Samples diagnosed as CIN II and CIN III, have a higher risk of proceeding to carcinoma in situ (CIS). For the past 20 years the presence of high-risk Human Papilloma Virus (HPV) genotypes has been associated with cervical dysplasia and its progression to cancer. This is now being used as an adjunct to detect cervical lesions in conjunction with Pap smear (Solomon et al, 2002).

The previous decade has witnessed the evolution of Fourier transform infrared (FTIR) spectroscopy as an independent modality to discriminate between diseased and normal tissue. Several comprehensive books and articles outlined the field (Pavia et al, 2001).

Early studies reported spectral differences between normal and malignant tissues by cytological and histological methods. The essential differences were related to changes in glycogen, nucleic acid, and protein content (Wood et al, 2004).

FTIR spectroscopy is used primarily for qualitative and quantitative analysis in chemistry and environmental sciences especially for determining the chemical
structure of many inorganic and organic compounds, drugs, pollutants, materials evaluation and identification. It is also used in deformulations, forensics purposes and measuring the level of purity of a compound (Wood et al, 2004).

This study presents preliminary results obtained from the analysis of 800 FTIR spectra obtained for cervical smears. The aim of this study is to demonstrate that this new technology can differentiate between different cytological results in cervical smears, and distinguish normal from dysplastic and neoplastic samples.

FTIR spectroscopy could be used an alternative to Pap smear, which is known to have low sensitivity. The clusters of spectra produced have the potential to be used as input data for the development of a diagnostic algorithm based on an Artificial Neural Network in future.

This study demonstrates that subtle differences between normal and cancer samples can be distinguished spectroscopically. The importance of this study is based on the fact that the spectroscopic changes precede the morphological changes of carcinogenesis which can be seen under the microscope.
1.1 Statement of the current problem

The shortage of trained cytopathologists, cytotechnologists and cytopathology laboratory facilities in developing countries, the long period (1-3 months) between the Pap screenings and the declaration of the test results (Jeronimo et al, 2005).

Pap test involves examination of the specimens under light microscope which is tedious, laborious and time-consuming. Highly skilled personnel are required as the reliability of the test depends upon human judgment.

The large variation in sensitivity between 30-80%, due to subjective observation was another problem limiting the success of screening program.

With these problems in mind, we embarked on an alternative method of screening using FTIR spectroscopy.
CHAPTER TWO

OBJECTIVES
OBJECTIVES

2.1 General objectives

The aim of this study was to evaluate the utility of a new, rapid, more cost-effective procedure for cervical cancer screening.

2.2 Specific objectives

1. To identify and characterize the infrared spectra of normal, pre-malignant (LSIL and HSIL) and malignant cervical scrapings.
2. To study the level of agreement between the results of FTIR spectroscopy with liquid based cervical smear cytology (gold standard).
3. To evaluate effectiveness of Fourier transform infrared FTIR spectroscopy in screening for pre-neoplastic lesions of cervical smears.

2.3 Hypothesis

FTIR spectroscopy is as effective as cervical cytology in detecting precursor lesions of cervical cancer.
CHAPTER THREE

LITERATURE REVIEW
Literature Review

3.1 Cervical Cancer

3.1.1 Background

In 2005, according to WHO projections, over 500,000 new cases of cervical cancer were diagnosed, of which over 90% were in developing countries. It is estimated that over 1 million women worldwide currently have cervical cancer; most of whom have not been diagnosed, or have no access to treatment that could cure them or prolong their life.

In 2005, almost 260,000 women died of the disease, nearly 95% of them in developing countries (WHO, 2006), making cervical cancer one of the gravest threats to women’s lives.

In many developing countries, access to health services is limited and screening for cervical cancer either is non-existent or reaches few of the women who need it. In these areas, cervical cancer is the most common cancer in women and the leading cause of cancer death among women (WHO, 2006).

3.1.2 Burden of suffering

Cervical cancer has a major impact on women's lives worldwide, particularly in developing countries where it is the leading cause of cancer deaths among
women. Four out of five new cases, and a similar proportion of deaths, occur in developing countries where screening programs are not well established or effective.

Experience in developed countries has shown that well planned, organized screening programs with high coverage can significantly reduce the number of new cases of cervical cancer and the mortality rate associated with it.

There is also evidence that general awareness about cervical cancer, effective screening programs, and the improvement of existing health care services can reduce the burden of cervical cancer for women and for the health care system. There is a huge difference in the incidence of and mortality from cervical cancer between developed and developing countries, as shown in figures 3.1 and 3.2 (WHO, 2006).

![Fig. 3.1 Age-standardized incidence rates of cervical cancer in developed and developing countries for the year 2005 (WHO, 2006)]
The hardest-hit regions are among the world's poorest. Central and South America, the Caribbean, sub-Saharan Africa, and parts of Oceania and Asia have the highest incidence rates over 30 per 100,000 women. These rates compare with no more than 10 per 100,000 women in North America and Europe.

Because the disease progresses over many years, an estimated 1.4 million women worldwide are living with cervical cancer, and two to five times more—up to 7 million worldwide—may have precancerous conditions that need to be identified and treated.
If it is not detected and treated early, cervical cancer is nearly always fatal. The disease, which affects the poorest and most vulnerable women, sends a ripple effect through families and communities that rely heavily on women's roles as providers and caregivers.

3.1.3 Etiology

3.1.3 (a) Human Papillomavirus (HPV)

Epidemiologic and clinical data indicate that HPV, especially HPV-16 and HPV-18, play a major role in the etiology of cervical cancer (De Marco et al, 2006). Expression of HPV-specific oncoproteins, E6 and E7, are considered essential in maintaining malignant growth of cervical cancer cells (Nubia et al, 2006).

However, HPV infections are widespread in the general population yet cannot be found in every patient with cervical cancer, nor do all infections with HPV result in cervical cancer. There are many investigators who argue that HPV is necessary etiologic factor, but few who maintain that it alone is sufficient to cause cervical cancer (Zur Hausen, 2002).

In the 1970s, Harald zur Hausen postulated a role for, and then found HPV-DNA in cervical cancers. In the 1980s, his group was the first to isolate HPV-16 and HPV-18 from cervical cancer tissues. Several epidemiologists have subsequently shown highly statistically significant associations between HPV and development of cervical intraepithelial neoplasia (CIN) grade 2 or 3, and with development of cervical cancer (Zur Hausen, 2002).
In 1995, a World Health Organization consensus panel collected biologic and epidemiologic data and concluded that at least HPV-16 and HPV-18 infection caused cervical cancer. HPV can be found in more than 90% of patients with cervical cancer worldwide, most frequently HPV-16 (50%), HPV-18 (12%), HPV-45 (8%), and HPV-31 (5%) (Schiffman and Brinton, 1995).

HPV is DNA tumor virus whose genome is organized in three regions: the early gene (E1 to E7), the late gene (L1 and L2) regions and the upper regulatory region (URR). The early and late gene regions are both protein-encoding, but the URR is non-encoding (Aggarwal et al, 2006).

The URR has numerous binding sites for many repressors and activators of transcription, and it may play a part in determining the range of hosts for specific HPV types.

In the protein-encoding regions, the E6 and E7 are considered to play the most major roles. These two units encode for oncoproteins that allow replication of the virus, increasing genomic instability, accumulation of oncogene mutations, further loss of cell-growth control, and ultimately cancer.

Both the E6 and E7 proteins alter the pathways that regulate tissue growth, by interfering with growth receptors or growth factors. Production of cytokines has been shown to be altered in cells infected with HPV-16. The E6 protein increases degradation of the p53 tumor suppressor protein, thereby interfering with apoptosis (programmed cell death).
The E7 protein disrupts complexes of the transcription factor E2F with the tumor-suppressor protein pRb and related proteins which are involved in control of the cell cycle, thus causing their degradation, altering control of transcription and progression of the cell cycle. The E7 protein has been shown to cause abnormal synthesis and duplication of centrosomes, resulting in abnormal mitotic division (Peter et al, 2006).

The late region units, L1 and L2 encode for viral capsid proteins during the late stages of virion assembly. The protein encoded by L1 is highly conserved among different papilloma virus species; therefore, antibodies against the bovine papilloma virus have been used to identify HPV capsid proteins in human tissues.

The minor capsid protein encoded by L2 has more sequence variations than that of the L1 protein; hence, antibodies against the L2 protein had been a source of antigen for specific types of HPV antibodies (Ho et al, 2006).

HPVs vary genetically not only between but also within types. Intra-type variants are defined as HPVs that vary by 2% or less in specified regions of the genome (Cheah, 1994).

Previous studies have reported intra-type sequence variations of the most common type, HPV-16, and have included specimens from different geographic areas. The major groupings of HPV-16 variants are designated European (E), Asian (As), Asian American (AA), African 1 (Af1), African 2 (Af2), and North
American 1 (NA1). Variants of these groups are denoted by HPV-16 E6 nucleotide position and substituted nucleotide (Wheeler et al, 1997).

### 3.1.3 (b) Sexually transmitted disease (STD)

The specific role of other infectious agents in the pathogenesis of cervical cancer has been investigated in many epidemiological surveys. The most studied sexually transmitted infectious agents in relation to cervical cancer are Herpes Simplex Virus (HSV-2), Chlamydia Trachomatis (CT) and Human Immunodeficiency Virus (HIV). Repeated exposure of the uterine cervix to the male partner's asymptomatic urogenital infection can lead to precancerous or cancerous lesions.

Zur Hausen was also the first to recognize that HPV was not sufficient for cancer induction, and proposed that HSV-2 and HPV act synergistically to induce cervical cancer (Zur Hausen, 2002).

Others have postulated a role for HSV-2 infection in the etiology of cervical cancer, while acknowledging the primary role for HPV, Hildesheim and colleagues studied women with invasive cervical cancer in Latin America and compared viral and behavioral characteristics with controls.

Compared with women negative for both HPV 16/18 and HSV-2, those positive for HSV-2 alone had a relative risk of 1.2. Those positive for HPV 16/18 DNA alone had a relative risk of 4.3 (95% CI=3.0, 6.0), and those positive for both
HSV-2 and HPV 16/18 had a relative risk of 8.8 (95% CI= 5.9, 13.0), suggesting a possible biological interaction. Furthermore, HSV-2 was persistent in a few selected cervical cancer tumors (Hildesheim et al, 1991).

Chlamydia trachomatis was associated with a two-fold increased risk for cervical cancer (OR=1.8; 95% CI, 1.2-2.7) in the large IARC multi-center case-control study. It was likely due to the inflammatory response with generation of free radicals and development of genetic instability (Smith et al, 2004).

Immunocompromised patients with HIV infection or organ transplantation are at high risk of HPV-associated anogenital cancers compared with age matched healthy individuals. HIV-positive women are at an increased risk of cervical cancer when compared with their HIV-negative counterparts.

This association appears to be stronger for women with a low CD4 T-lymphocyte count. As HIV infection is related to an immunocompromised state, it shows the importance of the host’s immunological cofactors in HPV carcinogenesis (Alcina et al, 2005).

3.1.3 (c) Nutritional factors

The few published studies assessing the role of diet on HPV persistence have shown a possible protective effect of diets rich in fruits, vegetables, Vitamins C and E, beta- and alpha-carotene. Evidence for a protective effect against cervical neoplasia was probable for folate, retinol, and Vitamin E, and possible
for vegetables, Vitamins C and B12, alpha-carotene, beta-carotene. Evidence for an increased risk of cervical neoplasia associated with high blood homocysteine was considered probable (Anna, 2000).

The current available evidence for an association between diet, nutritional status and cervical HPV carcinogenesis is not yet convincing, even though there is some support for the hypothesis that antioxidant nutrients may play a protective role in cervical carcinogenesis.

3.1.3 (d) Oral contraceptives and IUDs

The effects of oral contraceptives on the occurrence of cervical neoplasia are controversial (Smith et al, 2003). Cervical carcinogenesis may be hormone-dependent as judged by:

(a) the histological sensitivity of the cervix to hormonal influences
(b) the decreasing incidence of cervical cancer after menopause; and
(c) the results of animal experiments with estrogens and progesterones

Oral contraceptives cause atypical squamous epithelium, increase squamous metaplasia, basal cell and glandular hyperplasia of the cervix that could be misdiagnosed as malignant, but proved to be benign by using light and electron microscopy. Prolonged usage of an intrauterine device (IUD) is often associated with exfoliation of atypical cells that might be mistaken to represent carcinoma (Lech and Ostrowska, 2006).
3.1.3 (e) Genetic aspects

Several structural aberrations were observed in the chromosomes of patients, e.g. deletions and marked variability in size and shape of homologous chromosomes (Kate et al, 2004). The consistently observed chromosomal abnormalities in different stages of carcinoma of the cervix confirm the diagnostic value of the presence of such aberrations in cases of equivocal diagnosis. Thus, the presence of an abnormal clone of cells with chromosomal aberrations may indicate the presence of malignancy (Harry, 2005).

3.1.3 (f) Other risk factors

Tar-based vaginal douching may have a role with the development of cervical cancer. In 1931, Smith reported use of Lysol douches was significantly more common among cases than among controls. In 1950, Lombard and Potter discovered that douching with coal-tar derivatives was reported by more cervical cancers cases than controls (Lombard and Potter, 1950).

In 1967, Rotkin found a significant association between Lysol vaginal douches and cervical cancer compared with hospital-based controls matched for age, race, religion, and hospital (Rotkin, 1973).

Cigarette smoking also plays a role with cervical carcinogenesis. In 1977, Winkelstein reached a conclusion that cigarette smoking was a causative factor for cervical cancer (Harry, 2004). Other groups of investigators at John Hopkins
University have published data supporting passive cigarette smoking as a risk factor for cervical cancer (Winkelstein, 1977).

Another carcinogens generated by burning wood in kitchen stoves or ovens are tar-based compound and correlated with cervical cancer. In a follow-up study by a group of investigators, 125 women with CIN (CIN I, II, III) in Honduras were compared with 241 controls.

They concluded that chronic exposure to wood smoke significantly increased the risk of CIN III, and that chronic inhalation of carcinogens derived from wood smoke could have an effect on the progression to cervical cancer, similar to that observed from cigarette smoking (Winkelstein, 1977).

The natural history and risk factors that influence acquisition of persistent HPV infection that mediate progression in the continuum of cervical lesion grades is shown in the figure 3.3 (Bosch et al, 1997).
Normal Cervix

- HPV infection

LSIL

about 60% regress; 15% progress within 2-4 years

Coexisting factors:
- Smoking
- Oral contraceptive use
- Parity
- Other STDs
- Nutrition

HSIL

30% to 70% progress within 10 years

Invasive cervical cancer

Fig. 3.3 Diagram represents a current understanding of cervical cancer natural history (Bosch et al., 1997)
3.1.4 Epidemiologic Studies

The first observations relating to the incidence, distribution and possible causative factors of carcinoma of the uterine cervix were made in 1842 by Rigoni-Stern, an Italian physician who examined the records of deaths in Verona from 1760 to 1839. He noted that uterine cancer was less common in unmarried women and extremely rare in nuns (Hafez, 1982).

Uterine cancer was one of the most prevalent neoplasms reported in Western Europe during the mid 19th century. It is thought that the cervix was the primary site in most of these cases. An increased risk was noted in the lower socioeconomic classes, among multiparous women and in non-white women (WHO, 2002).

Previous studies on Jewish and non-Jewish populations conducted in USA, Europe indicated that Jewish women had a decreased incidence of cervical cancer despite being a part of the lower socioeconomic classes residing in New York City (Ezra et al, 2000).

The inference that circumcision of the Jewish males maybe a contributing factor prompted the examination of other ethnic groups such as Muslims, who also practice circumcision. The results of these investigations suggest that the variation in incidence between the compared ethnic groups cannot be attributed solely to circumcision but maybe due to associated cultural differences or other factors (Das et al, 2000).
The incidence of carcinoma of the cervix seems to be associated with low income and limited education, meanwhile in most countries of the Middle East conservative Muslim mode of life, male circumcision, sexual conservatism, and ritual hygiene probably operate in reducing incidence of carcinoma whereas aesthetic factors and complacency in seeking medical advice operate in delaying diagnosis (Fadua, 2001; Wang, 2004).

### 3.1.5 Cancer of the cervix in Malaysia

In 2004, the national Cancer Registry reported that cancer of the cervix was the second most common cancer among women in Malaysia for the year 2003 (Figure 3.4). It constituted 12.9% of total female cancers (NCR website, accessed on January 2006).

Cancer cervix was the eighth leading cause of death among medically certified deaths in 1998. Nearly 80% of patients with cervical cancer seen with advanced stages of IIB - IV A3. Despite the availability of screening program, 10.5% of female cancer deaths at government hospitals are due to cervical cancer, meanwhile 7.9% of all cancer admissions in government hospitals are diagnosed as cervical cancer (Othman, 2003).
There were a total of 1,557 cases of cancer cervix, with an ASR of 19.7 per 100,000 population (Table 3.1).

Table 3.1 Cervix uteri cancer incidence per 100,000 population (CR) and Age-standardized incidence (ASR), Peninsular Malaysia 2003 (Othman, 2003)

<table>
<thead>
<tr>
<th>Sex</th>
<th>No.</th>
<th>%</th>
<th>CR</th>
<th>ASR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1557</td>
<td>100</td>
<td>16.5</td>
<td>19.7</td>
</tr>
</tbody>
</table>
Cervical cancer incidence rate increased with age after 30 years. It has a peak incidence rate at ages 60-69 years, and declined thereafter (Figure 3.5).

![Fig. 3.5 Cervix uteri Age- specific cancer incidence per 100,000 population, Peninsular Malaysia 2003 (Othman, 2003)](image)

Chinese women had the highest ASR of 28.8 per 100,000 population, followed by Indians with ASR of 22.4 per 100,000 population and Malays with ASR of 10.5 per 100,000 population (Table 3.2).