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# TOLL-LIKE RECEPTOR 9 IN ALIMENTARY TRACT CANCERS

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*JOONAS KAUPPILA*

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***Abstract***

Cancers of the alimentary tract include many common cancer types, some of which have well-established treatment protocols and relatively good prognosis, such as colorectal cancer, and others, which have generally very poor prognosis. The gastrointestinal canal is colonized by a multitude of bacteria, the effects of which are currently poorly understood. Toll-like receptor 9 (TLR9) in cells of the alimentary tract recognizes the bacterial DNA-fragments and regulates immune functions in the host and the cancer.

This thesis examines the function and prognostic significance of Toll-like receptor 9 in oral and esophageal squamous cell carcinoma as well as in esophageal, gastric and colorectal adenocarcinoma. The studies were made using tissue samples from patient cohorts and various cell culture techniques. Our data indicate that high expression of Toll-like receptor 9 in cancer cells associates with metastatic properties in oral and esophageal cancers and poor prognosis in esophageal adenocarcinoma and oral squamous cell carcinoma. Cell culture studies further suggest that TLR9 is functional in alimentary tract cancers and mediates cellular invasion when activated.

Based on the results, TLR9 is active in alimentary tract cancers and its expression is related to poor cancer prognosis. Thus, TLR9 may represent a novel therapeutic target in alimentary tract cancers and might provide a link between bacteria and oral and gastrointestinal cancer.

*Keywords:* colorectal cancer, esophageal cancer, gastric cancer, innate immunity, matrix metalloproteinases, oral cancer, Toll-like receptor 9

**Kauppila, Joonas, Tolllinkaltainen reseptori 9 ruuansulatuskanavan syövissä.**
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Kirurgia; Diagnostiikan laitos, Patologia; Biolääketieteen laitos, Anatomia ja solubiologia; Medical Research Center; Oulun yliopistollinen sairaala
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## *Tiivistelmä*

Ruuansulatuskanavan syöpiin lukeutuu useita yleisiä syöpätyyppejä, kuten kohtalaisen hyväennusteinen paksusuolen syöpä, jonka hoitokäytäntö on vakiintunut. Toisissa ruuansulatuskanavan syövissä puolestaan ennuste on hyvin huono. Mahasuolikanavaa asuttavat moninaiset bakteerikannat, joiden vaikutuksia ymmärretään vielä kehnosti. Tollinkaltainen reseptori 9 (TLR9) tunnistaa näiden bakteerien DNA-rakenteita ja vaikuttaa yksilön ja syövän immuunivasteeseen.

Tämä väitöstutkimus selvittää TLR9:n toimintaa ja ennustevaikutusta suun ja ruokatorven levyepiteelisyövissä, sekä ruokatorven, mahalaukun ja paksusuolen adenokarsinoomassa. Tutkimus toteutettiin käyttäen syöpäpotilaiden kudosnäytteitä sekä soluviljelytekniikoita. Tuloksemme osoittavat, että TLR9:n lisääntynyt ilmentyminen syöpäsoluissa yhdistyy metastasointiin suu- ja ruokatorvisyövissä, sekä korkeaan kuolleisuuteen suun levyepiteelisyövässä ja ruokatorven adenokarsinoomassa. Soluviljelykokeidemme tuloksiin nojaten TLR9 toimii ruuansulatuskanavan syövissä ja sen aktivaatio saa aikaan solujen invasoitumisen.

Tutkimustuloksiimme vedoten TLR9 on aktiivinen ja toimiva ruuansulatuskanavan syövissä ja sen ilmentyminen liittyy huonoon ennusteeseen. TLR9 saattaa osoittautua uudeksi syöpähoitojen kohteeksi tai yhdistäväksi tekijäksi syövän ja bakteerien välillä ruuansulatuskanavan syövissä tulevaisuudessa.

*Asiasanat:* mahasyöpä, matriksin metalloproteinaasit, paksusuolen syöpä, ruokatorven syöpä, suusyöpä, synnynnäinen immuniteetti, Tollinkaltainen reseptori 9

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# Abbreviations

| | |
|---|---|
| APECED | Autoimmune polyendocrinopathy – candidiasis – ectodermal dystrophy |
| BE | Barrett's esophagus |
| CARD | Caspase recruit domain |
| CD | Cluster of differentiation |
| CDK | Cyclin dependent kinase |
| COX | Cyclo-oxygenase |
| CpG | Cytosine-phosphate-guanine |
| CRC | Colorectal cancer |
| EAC | Esophageal adenocarcinoma |
| EBV | Epstein-Barr virus |
| EC | Esophageal cancer |
| ER | Estrogen receptor |
| ESCC | Esophageal squamous cell carcinoma |
| GC | Gastric cancer |
| hCG | Human chorionic gonadotropin |
| HIF | Hypoxia-inducible factor |
| HIV | Human immunodeficiency virus |
| HPV | Human papilloma virus |
| HSP | Heat-shock protein |
| HSV | Herpes simplex virus |
| IL | Interleukin |
| IL-1R | Interleukin-1 receptor |
| IRAK | Interleukin-1 receptor-associated kinase |
| IRF | Interferon regulatory factor |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| MAMP | Microbe-associated molecular pattern |
| MMP | Matrix metalloproteinase |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NF-kB | Nuclear factor kappa beta |
| NLR | NOD-like receptor |
| NOD | Nucleotide-binding oligomerization domain |
| NSAID | Non-steroidal anti-inflammatory drug |
| NSCLC | Non-small-cell lung carcinoma |

| | |
|---|---|
| ODN | Oligodeoxynucleotide |
| OTSCC | Oral tongue squamous cell carcinoma |
| PAMP | Pathogen-associated molecular pattern |
| PR | Progesterone receptor |
| PRR | Pattern-recognition receptor |
| RCC | Renal cell carcinoma |
| RT-PCR | Reverse transcription polymerase chain reaction |
| siRNA | Short interfering RNA |
| SCC | Squamous cell carcinoma |
| SMA | Smooth-muscle actin |
| TGF-b | Transforming growth factor |
| TIMP | Tissue-inhibitor of metalloproteinase |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TNM | Tumor-lymph node-metastasis classification |
| TRAF | TNF receptor associated factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| VEGF | Vascular endothelial growth factor |
| WHO | World health organization |

# List of original publications

The thesis is based on the following articles, which are referred to in the text by their roman numerals:

I *Takala H, *Kauppila JH, Soini Y, Selander KS, Vuopala KS, Lehenkari PP, Saarnio J & Karttunen TJ (2011) Toll-like receptor 9 is a novel biomarker for esophageal squamous cell dysplasia and squamous cell carcinoma progression. J Innate Immun 3(6): 631–638.

II Kauppila JH, Takala H, Selander KS, Lehenkari PP, Saarnio J & Karttunen TJ (2011) Increased Toll-like receptor 9 expression indicates adverse prognosis in oesophageal adenocarcinoma. Histopathology 59(4): 643–649

III Kauppila JH, Karttunen TJ, Saarnio J, Nyberg P, Salo T, Graves DE, Lehenkari PP & Selander KS (2013) Short DNA sequences and bacterial DNA induce esophageal, gastric, and colorectal cancer cell invasion. APMIS 121(6): 511–522

IV Kauppila JH, Korvala J, Siirilä K, Manni M, Mäkinen LK, Hagström J, Atula T, Haglund C, Selander KS, Saarnio J, Karttunen TJ, Lehenkari PP & Salo T (2014) Toll-like receptor 9 (TLR9) mediates invasion and predicts prognosis in squamous cell carcinoma of the mobile tongue. Manuscript.

*Equal contribution

# Contents

# 1 Introduction

Cancers are one of the leading causes of death worldwide and according to the WHO, the number of cancer deaths is estimated to double in the near future. Despite recent advances in cancer treatments, carcinomas of the alimentary tract have a high mortality rate. They include many common cancer types, such as cancers of the colon and rectum, gastric carcinoma, esophageal carcinoma and oral carcinoma.

Shortly after birth, the human gastric epithelium is colonized with bacteria from maternal and environmental sources. Recent findings show that the human microbiome is by weight the largest organ in the body. Thousands of different species of bacteria reside in the alimentary tract. All of these bacteria contain CpG-sequences within their genome that are capable of activating TLR9, but interestingly, only some of these species have the ability to cause infections or cancer.

Toll-like receptors (TLR) are pattern-recognition receptors (PRR) in cells of the immune system. TLRs recognize various evolutionally conserved bacterial and viral components. These components include lipopolysaccharides of the bacterial cell wall, flagellin or DNA that contains certain sequences. TLRs can be found in cells of the innate and the adaptive immune systems, as well as in epithelial cells. TLR9, a microbial and endogenous DNA-recognizing Toll-like receptor, has been recently linked to cancer cell invasion. This endosomal receptor recognizes CpG-sequence containing oligonucleotides that have entered the cell via endocytosis. Invasion results in the activation of collagen-degrading proteins, called matrix-metalloproteinases (MMPs), and downregulation of tissue inhibitor of matrix metalloproteinase 3 (TIMP-3). Even though these *in vitro*-effects of TLR9 stimulation in breast and prostate carcinoma invasion have been well documented, not much is known about the real biological role of TLR9 in cancer.

The present study was designed to evaluate the expression and a possible mechanistic role of TLR9 in oral and gastrointestinal carcinomas. TLR9 and its downstream mediators were studied in cell lines and in clinical patient specimens of various cancers, including oral, esophageal, gastric and colon cancers.

# 2 Review of the literature

## 2.1 Innate immunity and pattern-recognition receptors

Innate immunity consists of the less-sophisticated mechanisms that defend the mammalian body from microbial and chemical attacks such as the skin, the mucous membranes, the acidity of the stomach and the flow of urine, as well as physiological mechanisms that detect pathogens and destroy them. These mechanisms include the complement system, antimicrobial agents secreted by cells and certain immune cells, such as monocytes, macrophages and dendritic cells. The aforementioned cells detect pathogens by means of pattern-recognition receptors (PRRs), which sense invariant, vital structures of pathogens, such as certain DNA structures or parts of the cellular membranes. (Janeway & Medzhitov 2002, Akira *et al.* 2006)

These pathogen-associated molecular patterns (PAMPs) are essential for the survival of the pathogens, as they include lipoteichoic acid (LTA) of the gram-positive bacteria, lipopolysaccharide (LPS) of the gram-negative bacteria, as well as flagellin from bacterial flagella, viral single-stranded and double-stranded RNA and bacterial DNA that contains the CpG-sequence. (Takeda *et al.* 2003, Akira *et al.* 2006) In addition to these and many other similar structures, the abbreviation "PAMP" is sometimes used in a broader sense to include damage-associated molecular patterns or DAMPs, which are endogenous, 'self'-structures recognized by PRRs. These endogenous and exogenous structures can also be summarized under microbial-associated molecular patterns (MAMPs), a term used more often in the context of plant immunity. (Mackey & McFall 2006)

PRRs can be classified into three groups by virtue of their function and localization. First, there is the soluble, opsonizing and complement-activating group, which includes lectins and other collectins as well as pentraxins such as C-reactive protein. (Holmskov *et al.* 2003, Nauta *et al.* 2003, Bottazzi *et al.* 2006, Fleer & Krediet 2007) Secondly there is the poorly understood group of endocytic receptors, such as the macrophage scavenger receptor (Mukhopadhyay & Gordon 2004). Thirdly, there exists the group of signaling PRRs that are membrane bound. These include Toll-like receptors (TLRs) and Nod-like receptors (NLRs), as well as caspase recruit domain (CARD) helicase proteins (Martinon & Tschopp 2005). When activated, these PRRs induce numerous inflammatory pathways and actions that enable the body to fight the infection or alternatively, to attack itself,

which generates an autoimmune reaction, autoimmune diseases or even cancer. (Akira *et al.* 2006, Fukata & Abreu 2008)

## 2.2 Alimentary tract and microbiome

The alimentary tract includes all organs from the mouth to the anus. The different parts share a common microbiology and a common basic structure. The inside of the tract is lined with a layer of epithelial cells covered by a mucus layer with underlying loose connective tissue, called the lamina propria, that contains numerous immune cells; the aforementioned are together called the mucosa. The gastrointestinal wall is composed of five layers: the mucosa; a thin layer of smooth muscle (the muscularis mucosa); loose connective tissue (the submucosa); layers of smooth muscle (the muscularis externa); and an outer layer of connective tissue (the serosa) (Young 2006).

The mucus layer contains glycoproteins, antimicrobial agents, salts and mostly water. The function of the mucus is to lubricate the intestinal lumen, but to also protect the underlying mucosa from harmful substances. Even with the mucus present, the intestinal wall is continuously in contact with intestinal bacteria and is thus the first line of defense against enteric antigens. This epithelial barrier regulates the passage of substances and bacteria through the epithelium by tight junctions at the boundary between the apical and basolateral membranes (Boleij & Tjalsma 2012).

Under the epithelium reside the leukocytes of the lamina propria, which form the second line of defense against the bacteria that translocate through the epithelial layer. Thus pathogenic bacteria encounter these leukocytes when epithelial barrier integrity is compromised and cannot straightforwardly enter the circulation and cause septic infections. It is also notable that enteric bacteria are actually needed for regulation of mucosal and systemic immunity. For example, germ-free mice did not produce as many circulating lymphocytes as did the conventional mice (Smith *et al.* 2007, Duerkop *et al.* 2009, Sansonetti & Medzhitov 2009). Feeding certain polysaccharides from commensal bacteria, *Bacteroides fragilis*, to the mice restored the immune system functions (Mazmanian *et al.* 2005).

The gastrointestine is normally colonized by commensal bacteria, which do not cause any diseases. The absolute number of bacteria outnumbers that of the cells of the host; the number of different bacterial species is estimated to be around 1000 (Boleij & Tjalsma 2012). According to the functional core

microbiome-hypothesis certain bacterial strains can be recovered from different individuals even when the total microbiome differs drastically between them (Rajilic-Stojanovic *et al.* 2009, Turnbaugh *et al.* 2009, Claesson *et al.* 2011). Host physiology and dietary factors affect the microbiome, but the microbiome can also affect the host. The intestinal bacteria digest xenobiotics and plant materials that are indigestible by humans, but they also produce many harmful and toxic components. In general, the microbiome is favorable for immunity and provides an intestinal barrier, but can also be harmful (Boleij & Tjalsma 2012).

## 2.3 Cancers of the alimentary tract

Gastrointestinal cancers, for example esophageal cancer, generally do not cause major symptoms in the early stages, and are commonly diagnosed late. Thus, these cancers have a poor prognosis and are difficult to treat. (Enzinger & Mayer 2003, Ferlay *et al.* 2010) The epidemiology of cancers in the gastrointestine varies geographically, as Asian countries have a higher incidence of esophageal squamous cell carcinoma and gastric carcinoma, while Western countries have a higher incidence of colorectal and other obesity-associated cancers (Parkin *et al.* 2005).

### *2.3.1 Carcinoma of the tongue*

Two and half percent of human cancers occur in the oral cavity. In 2000, approximately 300,000 primary oral cavity squamous cell carcinomas were reported worldwide. One third of the intraoral carcinoma diagnoses were accounted for by oral squamous cell carcinomas. The incidence of tongue cancer in males ranges from 0.4–9.4/100,000 persons per year, with the highest rates in Brazil, France and India, and the lowest in Northern Europe, females having a slightly lower incidence (Moller 1989, Parkin *et al.* 2002). The incidence of tongue cancer in Finland is increasing with the current incidence being around 1.2/100,000 (Finnish Cancer Registry). A similar trend is also being observed elsewhere in the world.

The main risk factors of oral cancers include tobacco and alcohol, which have a synergistic effect on the development of oral squamous cell carcinoma (Gillison 2007, Hashibe *et al.* 2007). Other risk factors include HPV-infection, poor oral hygiene and chewing of betel nuts (Moreno-Lopez *et al.* 2000, Herrero *et al.* 2003, Guneri *et al.* 2005, Hansson *et al.* 2005, Chen *et al.* 2008). No definitive

genetic risk factors have been found with the exception of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Goldstein *et al.* 1994, Rautemaa *et al.* 2007).

Even with the recent advances in surgical and radiotherapy techniques the five-year survival rate for tongue SCC remains around 50% (Sano & Myers 2007). The disease stage at the time of diagnosis, i.e. WHO TNM classification of squamous cell carcinomas, remains the best prognostic factor for tongue SCC. There are several histopathological scoring methods for prognostication, but none of them are in clinicopathological use (Anneroth *et al.* 1987, Bryne *et al.* 1989, Nathanson *et al.* 1989, Bryne *et al.* 1991, Bryne *et al.* 1992, Hiratsuka *et al.* 1997, Asakage *et al.* 1998, Brandwein-Gensler *et al.* 2005, Silveira *et al.* 2007, Sobin *et al.* 2010, Almangush *et al.* 2013). Among the histologic prognostic factors, many of the matrix metalloproteinases along with the alpha-SMA expression, which reflects the number of cancer-associated fibroblasts, appear promising, but require further studies (Korpi *et al.* 2008, Zhou *et al.* 2010, Bello *et al.* 2011, Makinen *et al.* 2011).

### *2.3.2 Esophageal cancer*

Esophageal cancer is the eighth most common cancer in the world with approximately 482,000 new cases worldwide in 2008. The incidence of esophageal cancer was 70/100,000 in 2008. The majority of esophageal cancers are esophageal squamous cell carcinomas (ESCC), but the incidence of esophageal adenocarcinoma (EAC) is rising rapidly (Parkin *et al.* 2005, Ferlay *et al.* 2010). In Finland, the incidences of esophageal cancer in 2009 were 39/100,000 and 12/100,000 persons per year among males and females, respectively. By 2009, of the total of 274 esophageal cancers, 47% were ESCCs and 37% were EACs (Finnish Cancer registry 2011).

As in the case of oral squamous cell carcinoma, tobacco and alcohol, low socioeconomic status, poor oral health and betel nuts, as well as the APECED-syndrome have been listed as risk factors for ESCC (Pickens & Orringer 2003, Garavello *et al.* 2005, Kamangar *et al.* 2006, Rautemaa *et al.* 2007, Kamangar *et al.* 2009). For EAC, the most important risk factor is Barrett's esophagus (BE), determined by columnar metaplastic cells in the distal esophagus showing intestinal metaplasia, which replaces the normal squamous epithelium after long-lasting gastroesophageal reflux, or gastroesophageal reflux disease (GERD). Patients with BE present a 30- to 125-fold risk for EAC compared to the normal

population (Fitzgerald 2006, Sikkema *et al.* 2010). A recent study, however, concluded that only 0.12% of patients with Barrett's esophagus develop EAC (Hvid-Jensen *et al.* 2011). Other minor risk factors include obesity, smoking, hiatal hernia and low socioeconomic status (Jansson *et al.* 2005, Corley *et al.* 2007, Holmes & Vaughan 2007, Abnet *et al.* 2008a, Corley *et al.* 2008, Kamangar *et al.* 2009). Furthermore, both cancers develop through dysplasia to cancer via genetic alterations. ESCC develops along a pathway from normal to dysplastic squamous epithelium and finally to squamous cell carcinoma. EAC is usually preceded by dysplastic changes in the columnar cell epithelium (glandular dysplasia), which further progress to invasive adenocarcinoma (Koppert *et al.* 2005, Cai *et al.* 2007).

The five-year survival rate for esophageal cancer varies from 10% to 16% (Parkin *et al.* 2005). According to the Finnish Cancer registry, the five-year cumulative relative survival ratios for EC patients in Finland were 10% for women and 11% for men (Finnish Cancer Registry 2011). After esophageal resection the five-year survival was 20.6% in a meta-analysis of Western populations (Hulscher *et al.* 2001). At the time of diagnosis, more than half of the patients have an inoperable disease (Shahbaz Sarwar *et al.* 2010).

The most important prognostic factor for esophageal cancers is the WHO TNM-classification (Sobin *et al.* 2010). Histologically defined grade of differentiation is also a predictor of prognosis (Liu *et al.* 2012). Various immunohistochemical biomarkers have been studied, but none of these are in routine use (Vallbohmer & Lenz 2006).

### *2.3.3 Gastric cancer*

Being the fourth most common cancer and the second most common cause of cancer death in the world, gastric cancer (GC) causes 700,000 deaths annually (Parkin *et al.* 2005). In 2006, 724 new gastric cancers were diagnosed in Finland (Finnish Cancer Registry 2011). Ninety percent of gastric cancers are adenocarcinomas, which originate from the columnar epithelium lining the stomach. The most notable risk factor for GC is infection with *Helicobacter pylori*, classified as a Class I carcinogen by the WHO. Other risk factors include smoking and eating smoked or salted foods (Ramon *et al.* 1993, Huang *et al.* 1998, Huang *et al.* 2000, Suerbaum & Michetti 2002, Kelley & Duggan 2003). Genetic predisposition for gastric adenocarcinoma occurs with mutations of

CDH1-, BRCA1- and BRCA2 genes (Semba *et al.* 1998, Johannsson *et al.* 1999, Grady *et al.* 2000, Jakubowska *et al.* 2002).

The gastric cancers can be classified into intestinal and diffuse subtypes, as described by Laurén in 1965 (Lauren 1965). The WHO system is derived from the Laurén classification, and categorizes the histologic patterns into five subtypes (namely adenocarcinoma including intestinal and diffuse types, papillary, tubular, mucinous and signet-ring cell) (Aaltonen *et al.* 2000). Other classification systems are those by Ming, Carniero and Goseki, but Laurén and WHO classifications are the most commonly used in clinical practice (Roy *et al.* 1998). Intestinal-type gastric carcinoma, which represents around 50–60% of GCs, is associated with *H. pylori* infection and diffuse-type GC, which in turn represents about 30–40% of GCs and is associated more with mutations and less with *H. pylori* (Lauren 1965, Hamilton *et al.* 2010). Intestinal-type GC bears better prognosis compared to diffuse-type GC and thus, the resection margins are suggested to be wider in the diffuse-type GC (Bozzetti *et al.* 1982, Dicken *et al.* 2005). Gastric cancer is usually detected in an advanced stage as a result of lack of symptoms (Hamilton *et al.* 2010). The five-year survival varies from 95% in patients with early stages to 10–30% in advanced stages (Keller *et al.* 2005). In Finland, the five-year survival rate for GC was 26% for females and 24% for males in the years 2003–2005 (Finnish Cancer Registry).

### *2.3.4 Colorectal cancer*

Colorectal cancer (CRC) is the third most common cancer in the world with about 1 million new cases diagnosed each year (Parkin *et al.* 2005). In Finland, CRC was diagnosed in 1,253 females and 1,387 males in the year 2010 alone (Finnish Cancer Registry). The majority of CRCs originate either through an adenoma-route or the recently established serrated route (Makinen *et al.* 2001, Hamilton *et al.* 2010). Multiple other developing pathways and different types of carcinoma are known, including mucinous adenocarcinoma, signet-ring cell carcinoma and medullary adenocarcinoma (Hamilton *et al.* 2010). The five-year survival of patients with colorectal adenocarcinoma ranges from over 60% in USA to 30% in India (Parkin *et al.* 2005).

The major risk factors for colorectal cancer are genetic, as almost 10% of CRCs are hereditary and approximately 14% are clustered in families, sporadic CRC accounting for 76% of CRC (Lynch & de la Chapelle 1999, Lynch & de la Chapelle 2003, de la Chapelle 2004). Other exogenous factors include eating red

meat and animal fat, smoking and alcohol. Eating vegetables, fruit or fibers, as well as using NSAID- or estrogen replacement therapy appear protective (Hamilton *et al.* 2010).

### *2.3.5 Bacteria and alimentary tract cancers*

There are numerous reports about the associations between bacteria and malignant transformation in the gastrointestinal organs. For example, poor oral hygiene increases the risk of esophageal and head and neck cancers, not to mention *H. pylori* in gastric carcinoma (Shiga *et al.* 2001, Suerbaum & Michetti 2002, Narikiyo *et al.* 2004, Michaud *et al.* 2007, Abnet *et al.* 2008b, Meurman & Uittamo 2008). Certain bacterial strains found in oral mucosa are associated with upper gastrointestinal malignancies (Narikiyo *et al.* 2004). In a normal esophagus, *Streptococcus* is the prevalent organism, but in patients with Barrett's esophagus or esophagitis a gram-negative anaerobic bacterial flora was more prevalent (Yang *et al.* 2009). There are no studies published about the effect of bacterial flora on esophageal cancers. In colorectal carcinoma, no definitive carcinogenic bacteria have been discovered, but in rodents the intake of probiotics has been associated with lower risk for colorectal carcinoma (Zhu *et al.* 2011). In recent studies, however, the *Fusobacterium nucleatum* has been linked to colorectal cancer (Castellarin *et al.* 2012, Kostic *et al.* 2012). Whether the *F. nucleatum* will be the *H. pylori* of the colon, is yet to be determined.

## 2.4 Toll-like receptors

Toll-like receptors are homologous to *Drosophila* Toll, first described by Christiane Nusslein-Volhard *et al.* in 1985, hence the name (Anderson *et al.* 1985). The Toll was found to be homologous to the Interleukin-1 receptor (IL-1R) in 1992 (Heguy *et al.* 1992). The immune function of Toll was noted in 1996, and its role in fighting against fungal infections was discovered by Hoffmann and colleagues. (Belvin & Anderson 1996, Lemaitre *et al.* 1996).

In mammals, the first TLR was described by Medzhitov *et al.* in 1997 (Medzhitov *et al.* 1997). The role of TLR4 as a receptor for lipopolysaccharide was later found by Beutler and colleagues (Poltorak *et al.* 1998). Jules Hoffmann and Bruce Beutler were later awarded the Nobel Prize in physiology or medicine in 2011 for their findings.

At present, 13 different mammalian TLRs are known, and at least ten of them are functional in humans (Chen *et al.* 2007, So & Ouchi 2010). TLRs are type I integral transmembrane glycoproteins that consist of extracellular, transmembrane and intracellular domains. The extracellular domain determines the ligand specificity and the intracellular domain executes signal transduction (O'Neill & Dinarello 2000, Akira *et al.* 2006, Matsushima *et al.* 2007, O'Neill & Bowie 2007).

**Table 1. TLR localization and examples of their respective ligands.**

| | Localization | Ligand (natural or synthetic) |
|---|---|---|
| TLR1 | Membrane | Triacyl lipopeptide (Takeuchi *et al.* 2002) |
| TLR2 | Membrane | Lipopolysaccharide (LPS) (Werts *et al.* 2001) |
| | | Lipoteichoic acid (LTA) (Schwandner *et al.* 1999) |
| | | Lipoproteins (Alexopoulou *et al.* 2002) |
| | | Zymosan (Ozinsky *et al.* 2000, Sato *et al.* 2003) |
| | | HSV-1 (Kurt-Jones *et al.* 2004) |
| TLR3 | Endosome | dsRNA (Alexopoulou *et al.* 2001) |
| | | ssRNA (Wang *et al.* 2002) |
| TLR4 | Membrane | LPS (Poltorak *et al.* 1998) |
| TLR5 | Membrane | Flagellin (Means *et al.* 2003) |
| TLR6 | Membrane | Diacyl lipopeptides (Takeuchi *et al.* 2001) |
| | | LTA (Ozinsky *et al.* 2000) |
| | | Zymosan (Ozinsky *et al.* 2000) |
| TLR7 | Endosome | ssRNA (Diebold *et al.* 2004, Lund *et al.* 2004) |
| | | Synthetic imidazolquinolines (Lee *et al.* 2003) |
| TLR8 | Endosome | ssRNA (Heil *et al.* 2004) |
| | | Synthetic imidazolquinolines (Jurk *et al.* 2006) |
| TLR9 | Endosome | Unmethylated CpG-ODNs (Hemmi *et al.* 2000) |
| | | Methylated CpG-ODNs (Ilvesaro *et al.* 2008) |
| | | Viral genomic DNA (Lund *et al.* 2003) |
| TLR10 | Membrane | Unknown |
| TLR11 | Membrane | Uropathogenic bacteria (Zhang *et al.* 2004) |
| | | Profilin (Yarovinsky *et al.* 2005) |
| TLR12 | Membrane | Unknown |
| TLR13 | Membrane | Ribosomal RNA (Oldenburg *et al.* 2012) |

Toll-like receptors reside on the endosomal membranes (TLRs 3 and 7–9) where they detect bacterial and viral nucleic acids, or on the cellular membranes (TLRs 1, 2, 4–6, and 9–13) where they detect, for example, components of microbial membranes (Akira *et al.* 2006). TLRs recognize various bacterial, viral, fungal

and protozoal, as well as synthetic and endogenous MAMPs, as summarized in Tables 1 and 2 (Poltorak *et al.* 1998, Schwandner *et al.* 1999, Takeuchi *et al.* 1999, Hemmi *et al.* 2000, Ozinsky *et al.* 2000, Alexopoulou *et al.* 2001, Li *et al.* 2001, Werts *et al.* 2001, Alexopoulou *et al.* 2002, Leadbetter *et al.* 2002, Takeuchi *et al.* 2002, Wang *et al.* 2002, Lee *et al.* 2003, Lund *et al.* 2003, Means *et al.* 2003, Sato *et al.* 2003, Kariko *et al.* 2004, Kurt-Jones *et al.* 2004, Lund *et al.* 2004, Zhang *et al.* 2004, Barrat *et al.* 2005, Coban *et al.* 2005, Yarovinsky *et al.* 2005, Akira *et al.* 2006, Jurk *et al.* 2006, Figueiredo *et al.* 2009, Oldenburg *et al.* 2012).

TLRs are found not only in the cells of adaptive and innate immune systems, but also in varying patterns in epithelial cells around the body (Schaefer *et al.* 2004, Fukata & Abreu 2008, Lim & Wang 2011, Mulder *et al.* 2012). Some TLRs have also been described in fibroblasts (Hasan *et al.* 2005, Mahanonda *et al.* 2007) and stem cells (Nagai *et al.* 2006, Nurmenniemi *et al.* 2010). Although interesting, the role of TLRs in adaptive immunity is beyond the scope of this review.

**Table 2. Examples of endogenous ligands of TLRs.**

| TLR | Endogenous ligand |
|---|---|
| TLR1 | Unknown |
| TLR2 | Heat Shock Proteins (HSPs) (Asea *et al.* 2002) |
| | Necrotic cells (Li *et al.* 2001) |
| TLR3 | mRNA (Kariko *et al.* 2004) |
| TLR4 | HSPs (Ohashi *et al.* 2000, Asea *et al.* 2002) |
| TLR5 | Unknown |
| TLR6 | Unknown |
| TLR7 | RNA immune complex (Pawar *et al.* 2006) |
| TLR8 | Unknown |
| TLR9 | Endogenous DNA (Barrat *et al.* 2005) |
| | Hemozoin (Coban *et al.* 2005) |
| | Chromatin-IgG-complex (Leadbetter *et al.* 2002) |
| TLR10 | Unknown |
| TLR11 | Unknown |
| TLR12 | Unknown |
| TLR13 | Unknown |

### 2.4.1 Toll-Like receptor 9

In 2000, the function of TLR9 as a CpG-oligodeoxynucleotide-recognizing receptor in splenic leukocytes was identified using TLR9-deficient mice (Hemmi *et al.* 2000). Knowledge about the role of TLR9 has since increased. TLR9 was first thought to discriminate between bacterial and self-DNA by methylation status, as unmethylated CpG-motifs are abundant in bacteria and relatively uncommon in vertebrates. (Hemmi *et al.* 2000) Later it was discovered that TLR9 recognizes CpG-motifs independent of methylation status and that the secondary and tertiary structures of DNA as well as certain hairpin-loops are more significant for TLR9 activation (Leadbetter *et al.* 2002, Ilvesaro *et al.* 2008). TLR9 was found to be expressed in immune cells, but later was discovered to be expressed in multiple epithelial cells, stem cells, fibroblasts, glial cells and muscle cells.

TLR9 resides in the endosomes and specifically recognizes CpG-motifs after unspecific endocytosis or endocytosis in immunocomplexes (Lande *et al.* 2007). Five different isoforms of TLR9 have been described, named TLR9-A, TLR9-B, TLR9-C, TLR9-D and TLR9-E. The functional differences between the isoforms have not been investigated but varying effects by different type CpGs on TLR9-mediated cytokine responses have been reported possibly caused by these isoforms. Current research has focused on measuring total TLR9 using antibodies targeted to exon 2 of TLR9, a common exon shared by all the isoforms (McKelvey *et al.* 2011).

Additionally, the 120kDa full length TLR9 appears to be inactive until cleaved from both C- and N-termini to a shorter form in the endolysosomal compartment (Park *et al.* 2008, Ewald *et al.* 2011). This cleavage is mediated by asparagine endoproteases and cathepsins(Ewald *et al.* 2011). The effects of cleavage on TLR9 function still needs further studies (Park *et al.* 2008).

### 2.4.2 TLR9 Signaling

Signaling of the TLR9 response is mediated mainly via Myeloid differentiation primary response gene 88 (MyD88) (Schnare *et al.* 2000). In leukocytes MyD88 can further activate different routes leading to induction of inflammation via IL-1R associated kinases 1 and 4 (IRAK-1 and IRAK-4) as well as interferon regulatory factor 7 (IRF-7), which causes recruitment of TRAF3 and TRAF6 (TNF receptor associated factors 3 and 6) (Lomaga *et al.* 1999, Suzuki *et al.*

2002, Kawai *et al.* 2004, Uematsu *et al.* 2005, Hacker *et al.* 2006). These changes ultimately cause activation of NF-κB and induction of type I interferons IFN-α and –β (Hoshino *et al.* 2002, Kawai *et al.* 2004, Uematsu *et al.* 2005). In breast carcinoma cells TRAF6 rather than MyD88 appears to mediate the CpG-induced invasion, downstream of TLR9 (Merrell *et al.* 2006).

## 2.5 TLR9 and matrix metalloproteinases

Matrix metalloproteinases (MMPs) are proteins that digest different components of the extracellular matrix, including collagens, fibronectins, laminins and proteoglycans (Bourboulia & Stetler-Stevenson 2010). The function of MMPs has been demonstrated in wound repair, cellular migration and cancer (Bourboulia & Stetler-Stevenson 2010). In cancer cells TLR9 activation seems to upregulate at least matrix metalloproteinases 2, 9 and 13 and downregulate the tissue inhibitor of metalloproteinases 3 (TIMP-3) (Merrell *et al.* 2006, Ilvesaro *et al.* 2007, Ilvesaro *et al.* 2008, Sandholm *et al.* 2011). This effect is mediated via TRAF6 and it is proposed that cancer cells might use this mechanism to facilitate invasion and metastasis (Ilvesaro *et al.* 2008). The simplified model of signaling is illustrated in Figure 1.

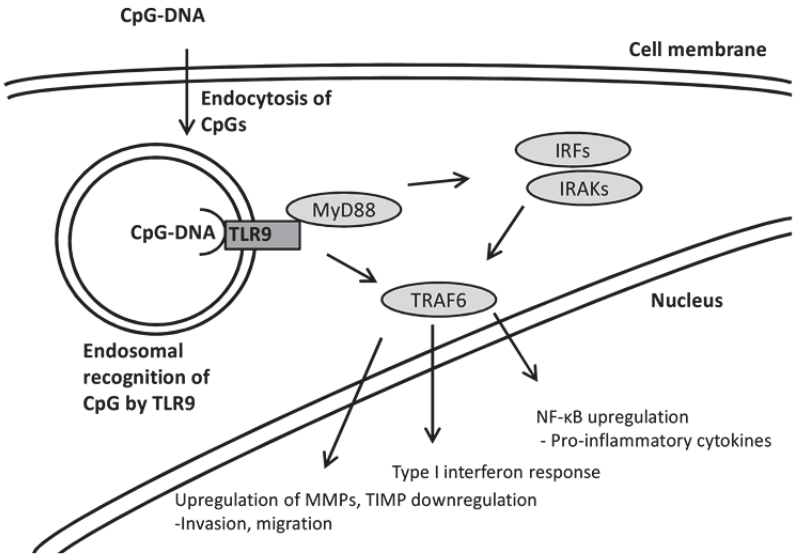


**Fig. 1. TLR9 signaling. CpG-DNA is endocytosed and recognized by TLR9 in the endosome. TLR9 signaling results in various responses by the cell illustrated in the figure.**

### *2.5.1 Matrix metalloproteinases 2, 9 and 13*

Matrix metalloproteinase 2 (MMP2) or Gelatinase A is a 72kDA type IV collagen-digesting enzyme. MMP2 along with the 92kDa matrix metalloproteinase 9 (MMP9), also called Gelatinase B (Liotta *et al.* 1979, Salo *et al.* 1983, Fessler *et al.* 1984), have both been shown to be important in cancer invasion and progression (Tryggvason *et al.* 1987, Stetler-Stevenson *et al.* 1993, Chambers & Matrisian 1997). Closely related by means of regulation and activation, both enzymes are secreted in a pro-MMP-form and then cleaved to a shorter, active form (Sternlicht & Werb 2001, Overall 2002).

Matrix metalloproteinase 13 (MMP-13) or Collagenase-3 was first found from breast carcinoma (Freije *et al.* 1994) and can be detected in various cancerous tumors and cells, such as breast, tongue, esophageal, gastric and brain cancers, but not in normal human tissues. High expression of MMP-13 has been linked to *in vitro* invasion as well as poor prognosis in cancers (Freije *et al.* 1994,

Etoh *et al.* 2000, Elnemr *et al.* 2003, Nyberg *et al.* 2003, Gu *et al.* 2005, Hodgson *et al.* 2009).

### *2.5.2 Tissue inhibitor of metalloproteinases-3*

Tissue inhibitor of metalloproteinases-3 is a 26kDa endogenous MMP inhibitor expressed in normal, healthy tissues. It has the ability to inhibit the activity of MMPs 1, 2, 3, 9 and 13 by binding to their active sites (Brew *et al.* 2000, Baker *et al.* 2002, Visse & Nagase 2003). Imbalance between MMPs and TIMPs are known to either cause or suppress tissue destruction and cancer metastasis (Schultz *et al.* 1988, Khokha *et al.* 1989, Clark *et al.* 1993, DeClerck & Imren 1994, Edwards *et al.* 1996). TIMP-3 has also been noted to cause apoptotic cell death by itself and not via MMPs (Brew *et al.* 2000).

## 2.6 Toll-like receptors and cancer

Various infections have been linked to cancer development, such as *H. pylori* and gastric cancer as well as EBV and hematologic cancers (Suerbaum & Michetti 2002, Thorley-Lawson & Gross 2004). Toll-like receptors recognize conserved components of bacteria and viruses, such as bacterial membranes, bacterial and viral DNA and fungal components, such as zymosan. TLRs are an essential part of the immune system, but are also expressed in various epithelial cells and stem cells (Nagai *et al.* 2006, Kawai & Akira 2010, Nurmenniemi *et al.* 2010).

TLR activation in the gut by bacteria is required for the development of immunity and intestinal homeostasis, as well as the regulation of epithelial proliferation (Mazmanian *et al.* 2005, Duerkop *et al.* 2009, Boleij & Tjalsma 2012). However, TLRs can also recognize many endogenous structures, such as mRNA, various proteins and DNA. As regulators of inflammation and intestinal epithelial proliferation, TLR signaling may have a pivotal role in carcinogenesis of carcinomas of the gastrointestinal tract (Fukata & Abreu 2008).

In 2006, Merrell *et al.* reported that activation of TLR9 with synthetic CpG-nucleotides induced invasion of breast cancer cells via matrix metalloproteinases (Merrell *et al.* 2006). After this finding, numerous reports concerning activation of different TLRs inducing invasion have been published. Invasion has been reported with activation of TLR2, TLR4 or TLR9 in breast cancer cells (Merrell *et al.* 2006, Xie *et al.* 2009, Liao *et al.* 2011). TLR5 activation induced invasion in salivary gland adenocarcinoma and TLR9 in many other cancers (Park *et al.*

2011). TLR stimulation of cancers has also been reported to cause immune evasion and apoptosis-resistance (Huang *et al.* 2005, Chiron *et al.* 2009). Surprisingly, TLR3 stimulation has also been reported to cause apoptosis in breast, prostate and head and neck cancer cells (Salaun *et al.* 2006, Paone *et al.* 2008, Nomi *et al.* 2010).

### *2.6.1 Toll-like receptor 9 and cancer*

TLR9 activation is known to induce cancer invasion. This is mediated by MyD88 via TRAF6 as well as by TRAF6 alone and executed by at least NF-κB (Chang *et al.* 2004, Merrell *et al.* 2006). It is known that in breast carcinoma cells, the stimulation of TLR9 results in induction of MMPs 2, -9 and -13 as well as downregulation of TIMP-3, which causes the invasion (Merrell *et al.* 2006, Ilvesaro *et al.* 2007, Ilvesaro *et al.* 2008, Sandholm *et al.* 2011, Wang *et al.* 2012, Tuomela *et al.* 2013a). In addition, cyclo-oxygenase-2 (COX-2) mediates invasion after stimulation of cancer cells with CpGs (Chang *et al.* 2005).

The TLR9-mediated invasion can be activated with multiple synthetic CpG-oligonucleotides, the most commonly used being the CpG-ODNM-362 in a nuclease-resistant phosphothioate backbone. In their studies in 2007 and 2008, Ilvesaro *et al.* showed that invasion via TLR9 can be also induced in prostate cancer cells with genomic DNA from *Escherichia coli* in a natural phosphodiester backbone and in breast cancer cells by unmethylated synthetic oligonucleotides that contain the CpG-sequence. Thus TLR9 appears to recognize DNA regardless of its methylation status (Ilvesaro *et al.* 2007, Ilvesaro *et al.* 2008). We have also demonstrated that TLR9 expression is downregulated in estrogen-receptor-positive (ER+) breast cancer cells and that ER as well as testosterone regulate TLR9 expression and signaling in triple-negative breast cancers *in vitro* (Sandholm *et al.* 2011).

At the present time, TLR9 expression has been demonstrated in multiple cancers and cancer cell lines, including esophageal squamous cell carcinoma, gastric cancer, glioblastoma, renal cancer, lung cancer, cervical cancer, oral cancer, head and neck cancers and pancreatic cancer. The *in vitro* studies concerning TLR9 in cancer cells are summarized in Table 3. (Chang *et al.* 2004, Chang *et al.* 2005, Droemann *et al.* 2005, Rayburn *et al.* 2006, Hasan *et al.* 2007, Ilvesaro *et al.* 2007, Rayburn *et al.* 2007, Ren *et al.* 2007, Bertin & Pierrefite-Carle 2008, Ilvesaro *et al.* 2008, Komine-Aizawa *et al.* 2008, Kundu *et al.* 2008, Assaf *et al.* 2009, Chiron *et al.* 2009, Di *et al.* 2009, Qiu *et al.* 2009, Ren *et al.*

2009, Xu *et al.* 2009, Berger *et al.* 2010, Brignole *et al.* 2010, Di *et al.* 2010, Tanaka *et al.* 2010, Wang *et al.* 2010, Xu *et al.* 2010, Min *et al.* 2011, Sandholm *et al.* 2011, Sinha *et al.* 2011, Weng *et al.* 2011, Wu *et al.* 2011) In clinical specimens of these cancers, increased tumor TLR9 expression has been associated mostly with poor prognosis, metastasis and/or increased proliferation. In renal cell carcinoma, as well as in triple negative breast cancer, the absence of TLR9 was associated with poor prognosis (Ronkainen *et al.* 2011, Tuomela *et al.* 2012). The expression of TLR9 and the main findings of these studies are summarized in the Table 4. (Lee *et al.* 2007, Ilvesaro *et al.* 2008, Jukkola-Vuorinen *et al.* 2009, Zhang *et al.* 2009, Zhou *et al.* 2009, Berger *et al.* 2010, Gonzalez-Reyes *et al.* 2010, Vaisanen *et al.* 2010, Wang *et al.* 2010, Gonzalez-Reyes *et al.* 2011, Hasimu *et al.* 2011, Min *et al.* 2011, Qiu *et al.* 2011, Ronkainen *et al.* 2011, Sheyhidin *et al.* 2011, Wu *et al.* 2011, Leng *et al.* 2012, Samara *et al.* 2012, Tuomela *et al.* 2012)

**Table 3. The effects of TLR9 stimulation in cancers in vitro.**

| Cancer | TLR9 agonist results in | Reference |
|---|---|---|
| Breast | Increased invasion | Merrell *et al.* 2006 |
| | Increased MMP-2, -9 and -13 expression | Merrell *et al.* 2006 |
| | Decreased TIMP-3 expression | Merrell *et al.* 2006 |
| | TLR9 activation regardless of ligand methylation | Ilvesaro *et al.* 2008 |
| | TLR9 activation negatively regulates ER-mediated proliferation | Qiu *et al.* 2009 |
| Breast | Protects cancer cells from TRAIL-induced-apoptosis | Chiron *et al.* 2009 |
| | Increased migration | Berger *et al.* 2010 |
| | ER-receptor alpha downregulates TLR9 and invasion | Sandholm *et al.* 2011 |
| | Testosterone upregulates TLR9 and invasion | Sandholm *et al.* 2011 |
| Chorion-carcinoma | Increased hCG production | Komine-Aizawa *et al.* 2008 |
| Colon | Decreased cancer cell survival | Rayburn *et al.* 2007 |
| | Upregulation of apoptosis | Rayburn *et al.* 2007 |
| | Downregulation of proliferation | Rayburn *et al.* 2007 |
| | Tumor cell autophagy | Bertin *et al.* 2008 |
| | Protects cancer cells from TRAIL-induced-apoptosis | Chiron *et al.* 2009 |
| Glial | Increased invasion | Wang *et al.* 2010 |
| | HIF-1α negatively correlated with TLR9 | Sinha *et al.* 2011 |
| Hepatic | Increased proliferation | Tanaka *et al.* 2010 |
| | Increased cancer cell survival | Tanaka *et al.* 2010 |
| | Increased chemoresistance | Tanaka *et al.* 2010 |
| Lung | Downregulation of apoptosis | Droemann *et al.* 2004 |
| | Invasion | Ren *et al.* 2007 |
| | Invasion in vitro | Xu *et al.* 2009 |
| | Invasion in vitro | Ren *et al.* 2009 |
| | Increased proliferation via CDK2 | Xu *et al.* 2010 |
| Neuroblastoma | Reduced proliferation, increased apoptosis via Caspases | Brignole *et al.* 2010 |
| | Increased apoptosis via Caspases | Brignole *et al.* 2010 |
| Oral | Increased proliferation | Min *et al.* 2011 |
| Pancreas | Reduced tumor cell activity | Wu *et al.* 2011 |
| Prostate | Downregulation of proliferation | Rayburn *et al.* 2006 |
| | Upregulation of apoptosis | Rayburn *et al.* 2006 |
| | Estradiol upregulates TLR9, Invasion via MMP-13 | Ilvesaro *et al.* 2007 |
| | NF-kb upregulation, carcinogenesis | Kundu *et al.* 2008 |
| | NF-kb upregulation, TGF-β and IL-8 upregulation | Di *et al.* 2009 |
| | NF-kb upregulation, COX-2 upregulation | Di *et al.* 2010 |
| Stomach | COX-2 activation, NF-kB activation | Chang *et al.* 2004 |
| Stomach | Invasion | Chang *et al.* 2005 |

| Cancer | TLR9 agonist results in | Reference |
|---|---|---|
| Uterine cervix | TLR9 promoter inactivation via HPV, TLR9 activation | Hasan *et al.* 2007 |
| | TLR9 increased chemosensitivity in HPV-negative cells | Weng *et al.* 2011 |
| Various | NF-kB-upregulation | Assaf *et al.* 2009 |

**Table 4. Studies of TLR9 in clinical cancer patient cohorts.**

| Cancer | TLR9* | High TLR9 correlated with | Reference |
|---|---|---|---|
| Breast | ++ | | Ilvesaro *et al.* 2008 |
| | ++ | ER-negativity, poor differentiation | Jukkola-Vuorinen *et al.* 2009 |
| | ++ | ER- and PR-negativity, poor differentiation | Berger *et al.* 2010 |
| | + | Good prognosis when TLR9+ in fibroblastoid cells | González-Reyes *et al.* 2010 |
| | + | Tumor size, lymph node metastasis, ER-negativity | Qiu *et al.* 2011 |
| | + | TLR9 negative patients had poor prognosis | Qiu *et al.* 2011 |
| | + | TLR9 negative TNBC patients had poor prognosis | Tuomela *et al.* 2013 |
| Esophageal | + | Good prognosis when TLR9+ in fibroblastoid cells | Sheydihin *et al.* 2011 |
| Glial | ++ | Poor prognosis | Wang *et al.* 2010 |
| | ++ | Poor prognosis, high MMP2 and MMP9 expression | Leng *et al.* 2012 |
| Lung | ++ | | Zhang *et al.* 2009 |
| | ++ | | Samara *et al.* 2012 |
| Oral | ++ | Tumor size, high tumor stage and high Ki67 | Min *et al.* 2011 |
| Ovary | 0 | | Zhou *et al.* 2009 |
| | ++ | Poor differentation | Berger *et al.* 2010 |
| Pancreas | ++ | | Wu *et al.* 2011 |
| Prostate | + | Poor differentation | Väisänen *et al.* 2010 |
| | + | Biochemical recurrence | González-Reyes *et al.* 2011 |
| Renal | + | TLR9 negative patients had poor prognosis | Ronkainen *et al.* 2011 |
| Uterine cervix | ++ | | Lee *et al.* 2007 |
| | ++ | Lymph node metastasis, HPV16 infection | Hasimu *et al.* 2011 |

* ++, upregulated in cancer; +, TLR9 expressed; 0, TLR9 not expressed.

### *2.6.2 TLR9, immunostimulation and cancer treatment*

Even before the discovery of TLR9 as their receptor, CpG-oligonucleotides have been demonstrated as possible vaccine adjuvants and anti-cancer agents because of their immunostimulatory activity on monocytes, dendritic cells, macrophages, B-, T- and NK-cells. The vaccination trials have been successful so far; CpG-ODN:s showed markedly improved immunization rates against hepatitis-B-virus, especially in immunocompromised HIV-infected patients. (Cooper *et al.* 2005)

CpG-ODNs have demonstrated efficacy in various settings of cancer therapy in murine models, especially when administered synergistically with other

treatments, including surgery, chemotherapy, antibodies and radiotherapy, as summarized in Table 5. (Pratesi *et al.* 2005, Damiano *et al.* 2006, Mason *et al.* 2006, Rayburn *et al.* 2006, Wang *et al.* 2006, Damiano *et al.* 2007, Meng *et al.* 2008, Ren *et al.* 2009, Xu *et al.* 2009, Brignole *et al.* 2010, Rosa *et al.* 2011, Sorrentino *et al.* 2011, Triozzi *et al.* 2011, Kim *et al.* 2012) CpG-ODN treatment has been vigorously tested against B-cell lymphomas in murine models, and it has been recognized as potentially effective in the long run because of their apoptosis-inducing effects. (Warren *et al.* 2000, Betting *et al.* 2009) Intravenous administration of CpG:s resulted in the induction of tumor-reactive $CD8^+$ T-cells in non-responders with non-Hodgkin's lymphoma in a small phase I study. (Brody *et al.* 2010) The efficacy of the CpG-treatments in B-cell lymphoma is yet to be evaluated in further clinical trials.

Clinical trials have been performed with a CpG-ODN PF-3512676 in late-stage non-small-cell lung carcinoma. In a phase II trial CpG-ODN-treatment in combination with standard chemotherapy increased the survival of patients non-significantly to 12.3 months compared to 6.8 months with chemotherapy. The response rate was also doubled compared to the standard chemotherapy. (Manegold *et al.* 2008) In both phase III trials the treatment with CpG-ODN increased the adverse effects and deaths, but did not increase survival or progression free survival. Thus the trials were terminated based on the recommendation of the trial Safety Committees. (Hirsh *et al.* 2011, Manegold *et al.* 2012) The published results concerning the TLR9 agonist PF-3512676 in phase II and phase III clinical trials in cancers of epithelial origin are summarized in the Table 6. (Pashenkov *et al.* 2006, Manegold *et al.* 2008, Thompson *et al.* 2009, Weber *et al.* 2009, Hirsh *et al.* 2011, Manegold *et al.* 2012)

In conclusion, TLR9-agonists have shown their efficacy as vaccine adjuvants and as a cancer treatment in various murine models, but in human cancers these results have not yet been duplicated. This could be explained either by the speculative functional differences in the immune systems of mice and human or the stimulating effects of CpG-ODNs on cancer invasion. Many of these studies were initiated or conducted at a time when tumor expression of TLR9 had not yet been demonstrated. Thus, possible direct tumor cell effects of the CpG-ODNs were not necessarily taken into account when the trials were designed. Thus, the possible cancer invasion-inducing effects of CpG-ODNs in these trials have not been addressed. It is also possible that the patient selection for these studies has been suboptimal.

**Table 5. The effects of TLR9 in epithelial or connective tissue malignancies in murine xenografts.**

| Cancer | Effect of TLR9 agonist | Reference |
|---|---|---|
| Breast | Increased chemo- and radiotherapy efficacy | Mason *et al.* 2006 |
| Colon | Antitumor effect when combined with EGFR-inhibitor | Damiano *et al.* 2006 |
| | Increased bevacizumab efficacy | Damiano *et al.* 2007 |
| | Tumor promotion in CEA-transgenic mice | Triozzi *et al.* 2011 |
| | Inhibition of tumor growth with cetuximab | Rosa *et al.* 2011 |
| | Inhibition of tumor metastasis | Kim *et al.* 2012 |
| Fibrosarcoma | Increased chemo- and radiotherapy efficacy | Mason *et al.* 2006 |
| Glial | No TLR9 expression in xenografts | Meng *et al.* 2008 |
| Lung | Increased apoptosis | Wang *et al.* 2006 |
| | Decreased proliferation | Wang *et al.* 2006 |
| | Increased chemotherapy efficacy | Wang *et al.* 2006 |
| | Increased metastasis | Xu *et al.* 2009 |
| | Increased metastasis | Ren *et al.* 2009 |
| | Increased metastasis and production of VEGF-A | Sorrentino *et al.* 2011 |
| Neuroblastoma | Prolonged survival | Brignole *et al.* 2010 |
| Pancreas | Prolonged survival when combined with TLR9-agonist | Pratesi *et al.* 2005 |
| | Inhibition of tumor growth with cetuximab | Rosa *et al.* 2011 |
| Prostate | Tumor regression | Rayburn *et al.* 2006 |
| | Increased chemotherapy efficacy | Rayburn *et al.* 2006 |

**Table 6. Published phase II or III clinical trials concerning TLR9 agonist PF-3512676 in treatment of cancers of epithelial origin.**

| Cancer | Phase | Patients | Study model | Adminstration frequency | Conclusion | Main result | Reference |
|---|---|---|---|---|---|---|---|
| Melanoma | II | 20 | 6mg s.c. weekly for 24 weeks | weekly for 24 weeks | TLR9 agonist is safe and tolerated | 2 patients had partial response | Pashenkov *et al.* 2006 |
| Melanoma | II | 184 | 10mg / 40mg s.c. and/or dacarbazepine | 10mg or 40mg every 3 weeks | TLR9 agonist is safe and tolerated | TLR9 agonist did not improve response | Weber *et al.* 2009 |
| NSCLC | II | 111 | 0.2mg s.c. | on days 8 and 15 every 3 weeks | TLR9 agonist improves response when combined with chemotherapy | 1-year survival slightly improved with TLR9-agonist | Manegold *et al.* 2008 |
| RCC | II | 39 | escalating s.c. up to 0.81mg/kg | | TLR9 agonist is safe and tolerated | 2 patients had partial response | Thompson *et al.* 2009 |
| NSCLC | III | 839 | cisplatin + gemcitabine with or without TLR9-agonist 0.2mg/kg s.c. | on days 8 and 15 every 3 weeks | Study terminated due to safety issues | Increased adverse events, no improvement in response | Manegold *et al.* 2012 |
| NSCLC | III | 828 | paclitaxel + carboplatin with or without TLR9-agonist 0.2mg/kg s.c. | on days 8 and 15 every 3 weeks | Study terminated due to safety issues | Increased adverse events, no improvement in response | Hirsh *et al.* 2011 |

NSCLC, non-small cell lung cancer; RCC, Renal cell carcinoma

### 2.6.3 TLR9 in normal and neoplastic alimentary tract

TLR9 has been demonstrated in the normal epithelium of all organs of the gastrointestinal canal, including oral, esophageal, gastric and small and large intestinal epithelium. The function and relevance of TLR9 has been studied mostly in gastric and colonic epithelium, no reports at all being available from esophageal epithelium.

In a study by Min *et al.* TLR9 protein expression was shown to be increased in oral squamous cell carcinoma when compared with normal controls. In their study with a fairly small patient population they found that increased TLR9 expression correlated with higher tumor stage and increased proliferation. Treatment of oral squamous cell carcinoma cells with CpG-ODN was also demonstrated to activate proliferation and increase expression of various interleukins. (Min *et al.* 2011)

In a study by Sheydihin *et al.* TLR9 mRNA was expressed in 15% of the normal esophagi and protein in 4.6% of the normal esophagi of the patients with ESCC. The numbers for ESCC were 55% and 74.2%, respectively. In the study, increased TLR9 protein expression was associated with poor differentiation of the cancer. They also noted that high fibroblast TLR9 expression indicated a lower probability of metastasis. (Sheyhidin *et al.* 2011)

In a large study consisting of 1408 patients, polymorphisms of TLR9 were not associated with esophageal or gastric cancers. (Hold *et al.* 2009) Most of the studies concerning TLR9 and gastric cancer have been conducted to explore the relationship of *H. pylori* and gastric cancer. *H. pylori* isolates induced COX-2 expression via a TLR9-dependent pathway in AGS-cells (Chang *et al.* 2004). This mechanism was also demonstrated to mediate invasion in gastric cancer cells and vary between the *H. pylori* strains used (Chang *et al.* 2005). In an immunohistochemical study TLR9 expression was demonstrated in 6 of 22 gastric adenocarcinomas and all of the normal gastric epithelia. In this study, no TLR9 expression was present in the 10 intestinal metaplasias or the three gastric dysplasias. (Schmausser *et al.* 2005)

TLR9 expression has been demonstrated in the normal small intestine, but not in the cancers located there. TLR9 signaling has been thought to be associated with inflammatory bowel disease and thus probably with the cancers of the small intestine. (Rumio *et al.* 2004)

Expression of TLR9 has been well characterized in colonic epithelium, but not in the cancers of the colon. In a model of colonic epithelium TLR9 expression was polarized, causing a decrease in inflammatory cytokines when activated from the apical side but an increase in inflammation when activated from the basolateral side. The induction of inflammatory cytokines resulted from activation of NF-kappaB. When the cells were stimulated basolaterally, IkappaB was activated (Lee *et al.* 2006). The exact signaling path was not characterized. A TLR9 polymorphism has been linked to Crohn's disease, which was associated with disease severity. (Torok *et al.* 2004, Torok *et al.* 2009) NF-kappaB-signaling has been linked to colonic carcinogenesis (Vaiopoulos *et al.* 2010) and is an important endpoint in TLR9 signaling as well, so further studies are needed to confirm the role of TLR9 in intestinal carcinogenesis.

In a study by Eiró *et al.* immunohistochemical TLR9 expression of colonic polyps was studied. (Eiro *et al.* 2012) They compared the expression of TLRs between colorectal polyps, which produced a cancer later to those without subsequent tumor development. TLR9 was increased in all polyps when compared to normal epithelium and in hyperplastic and adenomatous polyps when compared to other polyps. TLR9 mRNA and protein expression was lower in hyperplastic and villous adenoma polyps, which developed into a carcinoma when compared to the polyps without cancer development.

Taken together, the links between TLR9 and intestinal carcinogenesis remain speculative. Increased TLR9 expression has been connected to various factors of adverse prognosis in various cancers, but no studies with large patient populations have been published yet.

# 3 Aims of the study

The main aim of this study was to further characterize the expression of TLR9 and the invasive function mediated via TLR9 in oral and gastrointestinal carcinomas. More specifically, the objectives were:

1. To characterize TLR9 expression patterns in normal esophageal and lingual epithelium, as well as in esophageal squamous dysplasia.
2. To describe the expression of TLR9 and its relations to clinicopathological variables in clinical cohorts of esophageal adenocarcinoma, esophageal squamous cell carcinoma and oral squamous cell carcinoma patients.
3. To investigate the effect of TLR9 stimulation on gastrointestinal and oral cancer cell invasion *in vitro* and determine the effects of TLR9 activation on matrix metalloproteinase 2, 8, 9 and 13 expression, as well as TIMP-3 expression, using synthetic TLR9-ligands that contain the CpG-sequence.
4. To compare the invasive effects caused by TLR9 stimulation by synthetic DNA ligands, short DNA sequences and bacterial DNA in gastrointestinal cancer cell lines *in vitro*.

# 4 Materials and methods

The materials and methods used in the thesis are summarized in tables 7 and 8 below. Detailed information with references is found in the original papers I-IV.

Samples of the study patients were obtained from the archives of the Department of Pathology, Oulu University Hospital, Oulu, Finland (I, II, IV) and Helsinki University Central Hospital, Helsinki, Finland (IV). The study cases were reviewed from the hematoxylin-eosin slides for the correct diagnosis and histological properties by an expert pathologist. Clinical data was obtained from patients' clinical charts and survival data from Statistics Finland (Helsinki, Finland). The study and data inquiry were approved by Oulu and Helsinki University Hospital Ethical Committees and the National Committee of Medicolegal Affairs (VALVIRA). The patient data from the studies is summarized in table 8.

**Table 7. Methods used in the original publications.**

| Level | Methods | Used in |
|---|---|---|
| RNA | RNA isolation | III |
| | quantitative RT-PCR | III |
| Protein | Protein isolation | III, IV |
| | Western blotting | III, IV |
| Cells and tissues | Preparation and staining of tissue sections | I, II, IV |
| | Light microscopy | I,II, III, IV |
| | Computerized quantification of immunostaining | I, II |
| | Assessment of apoptosis | I, II |
| | Assessment of vascular density | I, II |
| | Assessment of proliferation | I, II |
| | Cell culture | III, IV |
| | Invasion assay | III, IV |
| | Cell viability assay | III |
| | RNA interference | IV |
| | Myoma organotypic model | IV |
| | Wound healing assay | IV |
| Other | Statistical analysis | I, II, III, IV |

**Table 8. Summary of the patients in studies I, II and IV.**

| Study | I | II | IV |
|---|---|---|---|
| Diagnosis | ESCC | EAC | OTSCC |
| Patient no | 51 | 85 | 131 |
| Samples analyzed | 46 | 76 | 119 |
| Median age (years) | | 61 | 65 |
| Median follow-up (months) | | 17.5 | 119 |
| Age at the time of diagnosis | | | |
| <60 | 9 | 25 | 42 (<55 yrs) |
| 60–65 | 9 | 24 | 36 (55–70 yrs) |
| >65 | 33 | 28 | 53 (>70 yrs) |
| Sex | | | |
| Male | 23 | 63 | 62 |
| Female | 28 | 16 | 69 |
| Tumor grade (differentiation) | | | |
| 1 (Good) | 3 | 38 | 49 |
| 2 (Moderate) | 27 | 21 | 67 |
| 3 (Poor) | 18 | 21 | 15 |
| Tumor Stage | | | |
| I | 3 | 17 | 71 (I-II) |
| II | 20 | 29 | |
| III | 14 | 10 | 60 (III-IV) |
| IV | 9 | 27 | |
| Lymph nodes | | | |
| Negative | 10 | 39 | 90 |
| Positive | 36 | 44 | 41 |
| Metastasis at the diagnosis | | | |
| No | 37 | 57 | 131 |
| Yes | 9 | 25 | 0 |

# 5 Results

## 5.1 Occurrence of TLR9 in normal epithelia and cancers of the study

We measured TLR9 expression on the mRNA and/or protein level in all the cell lines (OE33, AGS, CaCo-2, MDA-MB-231, SCC-15, SCC-25, HSC-3) studied. TLR9 was expressed in all 24 normal esophageal epithelia studied in a gradually decreasing pattern from basal cells towards the apical cells (I). In normal tongue, TLR9 was expressed in 112 of the 115 cases (IV). Of the tumors, TLR9 was expressed in all 46 of the esophageal squamous cell carcinomas (I), all 76 of the esophageal adenocarcinomas (II) and 181 of the 195 oral tongue squamous cell carcinomas (IV). The intensity of the staining was higher in cancer cells in both the ESCC and OTSCC compared to normal epithelium. (I, IV)

## 5.2 TLR9 activation induces cancer invasion and migration

Activation of TLR9 with stimulatory CpG-ODN resulted in a significant increase in cellular invasion in all of the cell lines studied (III, IV). We also used synthetic 9-mer and G-quadruplex DNA in a phosphodiester backbone and genomic DNA from *Escherichia coli* and *Helicobacter pylori* to stimulate TLR9 in MDA-MB-231, OE33, AGS and CaCo-2 cell lines. We observed an increase in invasion in all of the cell lines with these ligands (III). The invasion could be inhibited with the TLR9 antagonist chloroquine and a matrix metalloproteinase inhibitor GM-6001 (III).

Downregulation of TLR9 through siRNA reduced the effect of CpG-ODN on oral cancer cell invasion compared to cells treated with PBS or non-targeting siRNA. CpG-ODN treatment also modestly induced invasion in a myoma organotypic model in HSC-3 oral cancer cells. Migration was induced in HSC-3 cells in a wound healing assay by CpG-ODN treatment and this effect was decreased by TLR9- or MMP-13-neutralizing antibodies. (IV)

## 5.3 Activation of TLR9 results in changes in matrix metalloproteinase-2, -9 and -13 and TIMP-3 expression

Activation with different ligands resulted in cell-specific changes in TLR9, MMP2, MMP9, MMP13 and TIMP3 mRNA levels in MDA-MB-231-, OE33-, AGS- and CaCo-2 cells. Upon stimulation, TLR9 was up-regulated in MDA-MB-231- and AGS cells, but down-regulated in OE33-cells (III).

After stimulation, MMP-2 expression was up-regulated in AGS cells, down-regulated in MDA-MB-231 cells and not expressed in OE33 cells. MMP-9 was expressed in MDA-MB-231- and OE33 cells and up-regulated upon stimulation by different ligands. In CaCo-2 cells stimulation caused MMP-9 downregulation and in AGS cells MMP-9 was not expressed. MMP-13 was up-regulated in MDA-MB-231-, OE33- and AGS cells upon stimulation, whereas in CaCo-2 cells MMP-13 levels were left unchanged. TIMP-3 was down-regulated in CaCo-2 cells and unchanged in MDA-MB-231-, and AGS cells. OE33 cells did not express TIMP3 mRNA (III). The mRNA analysis is summarized in the table in original publication III.

## 5.4 TLR9 in clinical materials

### *5.4.1 Esophageal squamous dysplasia*

TLR9 was expressed in all 12 of the esophageal high grade dyplasias assessed. When compared to the adjacent normal epithelium, an obvious increase in TLR9 intensity could be observed, the expression extending to the apical side of the epithelium (I).

### *5.4.2 Neoplasia*

In squamous cell carcinoma, high TLR9 expression was associated with poor differentiation, positive lymph nodes and distant metastasis (I). In esophageal adenocarcinoma, we observed significant associations with high TLR9-expression and high pT stage, poor differentiation, lymph node metastases, metastatic disease and high proliferation (II). In OTSCC, high TLR9 expression was associated with poor histologic grade and high expression of matrix metalloproteinase 13 (IV).

### *5.4.3 Prognostic aspects*

In ESCC we were unable to demonstrate any prognostic value for TLR9 (I). In EAC, high TLR9 expression was associated with poor survival (II). In OTSCC, high TLR9 expression was a predictor of poor prognosis and shorter disease-free survival when standardized by age, tumor stage and grade of differentiation (IV).

# 6 Discussion

In this study, we explored the effect of TLR9 stimulation in gastrointestinal cancer cell lines in vitro, as well as the expression of TLR9 in oral and esophageal carcinoma patient cohorts. TLR9 expression was further compared with standard clinicopathological parameters associated with the specimens. The prognostic significance of the immunohistochemical expression of TLR9 in different carcinomas of the upper gastrointestinal tract was also studied. It was shown that TLR9 is expressed in various cancerous cells and tissues of alimentary tract origin. Thus, TLR9 appears to be a promising factor in the assessment of alimentary tract cancer prognosis. Understanding the role of TLR9 in pathophysiology of alimentary tract cancers may reveal new treatment possibilities in cancers of the alimentary tract.

## 6.1 TLR9 expression and effect in alimentary tract cancers

### *6.1.1 TLR9 affects squamous cell carcinoma of the tongue, invasion, migration and progression*

TLR9 was expressed in normal tongue, as well as in OTSCC. High TLR9 expression was associated with MMP-13 expression and poor cellular differentiation, as well as with poor cancer-specific survival. In vitro, we found that TLR9 indeed mediates cellular invasion of OTSCC, as TLR9 stimulation by CpG-ODNs induced invasion and migration in such cancer cells

TLR9 has been shown to be expressed in normal tongue and Lichen planus in studies by us and Min *et al.* (Min *et al.* 2011, Siponen *et al.* 2012) Min. et al confirmed that TLR9 expression was associated with tumor size and stage, as well as with increased proliferation. (Min *et al.* 2011) This is contradictory to our results, as we did not find any associations with these properties, but rather with poor histological grade. In our study, we did not assess proliferative effects of TLR9 ligands. Min *et al.* however, activated OTSCC cells with CpG-ODNs and observed increased proliferation in these cells by induction of Cyclin D1. (Min *et al.* 2012)

Taken together, the present research results and other reports suggest that TLR9 may play a role in oral carcinoma proliferation, invasion and progression.

### 6.1.2 TLR9, a player in esophageal cancer carcinogenesis

In study I we also found that TLR9 is more strongly expressed in esophageal dysplasia as compared with normal epithelium. This novel result suggests that TLR9 could be used as an immunohistochemical biomarker of esophageal dysplasia. However, no samples of esophagitis were included, which is the main differential diagnostic problem of esophageal dysplasia. Taken together, our results call for a study with more extensive patient material, including esophagitis samples, to be conducted. Such further studies are needed to determine the sensitivity and specificity of TLR9 as a biomarker of esophageal squamous cell dysplasia. In ESCC, high TLR9 expression was associated with clinical variables indicating poor prognosis, such as cancer metastasis and increased proliferation. (I)

In study II we studied esophageal adenocarcinomas and their TLR9 expression. We found that high TLR9 expression was associated with advanced stage, lymph node metastasis, poor differentiation and high proliferation in esophageal adenocarcinoma. (II) TLR9 was expressed in the OE33 esophageal adenocarcinoma cell line, and when these cells were stimulated with CpG-ODN or other CpG-containing DNA, MMP-9 and MMP-13 mRNA expression were induced. TLR9 stimulation caused invasion in OE33 cells, which could be inhibited by chloroquine and matrix-metalloproteinase inhibitor. (III)

Before this study was undertaken, there were no publications describing TLR9 expression in esophageal cancer, squamous cell or adenocarcinoma. The only study on TLR9 expression in esophagus was conducted by Sheydhin *et al.* where they characterized the expression of TLR9 in 87 ESCC patients. (Sheyhidin *et al.* 2011) The results were partly in line with study I, as TLR9 was basally expressed in normal esophagus and increased in poorly differentiated tumors. Contradictory to our study, they did not find any associations between lymph node metastasis and TLR9 in cancer cells. They did show, however, that low TLR9 expression of cancer-associated fibroblasts was associated with tumor T-stage and lymph node metastasis of ESCC. (Sheyhidin *et al.* 2011)

Taken together, these studies suggest that TLR9 may have a significant role in esophageal carcinogenesis. Whether it is the bacteria that we swallow every day that cause TLR9-dependent reactions in the epithelium or whether these are secondary to cancerous transformation, remains to be investigated.

### 6.1.3 TLR9-dependent effects in gastric cancer produced by DNA ligands

In study III we detected induced invasion in AGS cells when stimulated with CpG-ODNs but also with *H. pylori* DNA. These ligands induced MMP2 and MMP13 mRNA expression. The invasion itself was dependent on endosomal signaling (as inhibited by chloroquine) and matrix metalloproteinases.

In the study by Chang *et al.* gastric carcinoma cells were cocultured with *H. pylori*, which led to increased invasion and angiogenesis in AGS cells and another gastric cancer cell line, MKN45. They showed that this invasion is COX-2 dependent via various transcription factors, but the effect of *H. pylori* was mediated through TLR2 and TLR9. (Chang *et al.* 2005) In the study by Schmausser *et al.* TLR9 expression was lost in most dysplasias and adenocarcinomas of the stomach. When present, the normal basolateral polarization of TLR9 was deranged in the gastric dysplasia-adenocarcinoma-sequence. (Schmausser *et al.* 2005)

Taken together, our and others' results suggest that TLR9 may recognize bacterial, for example *H. pylori*, DNA and induce invasion.

### 6.1.4 Bacteria and TLR9, the future of colorectal cancer research?

Colorectal cancer develops in the area most densely populated by bacteria in humans, the colon. As a bacterial DNA receptor, it is likely that TLR9 has an important role in colonic homeostasis and carcinogenesis. In study IV we stimulated colorectal cancer cells with CpG-ODNs, which resulted in invasion of colorectal cancer cells, as well as matrix metalloproteinase upregulation. Invasion was also induced with bacterial DNA and natural phosphodiester-backbone-containing DNA representing endogenous DNA.

Eiró *et al.* studied TLR9 in colonic polyps, but not in colorectal cancer. They discovered that TLR9 expression was decreased in hyperplastic polyps that later developed colorectal carcinoma. Similar results were not obtained in other types of polyps. However, all types of polyps studied expressed TLR9. This might indicate that the normal function of TLR9 is different from the function of TLR9 in cancers. (Eiro *et al.* 2012)

These results combined with the fact that there was genetic material from *Fusobacterium nucleatum* found in colorectal cancer specimen, warrants a series of studies to evaluate the role of TLR9 or other bacterial pattern-recognition

receptors in colorectal cancer carcinogenesis. (Castellarin *et al.* 2012, Kostic *et al.* 2012)

### *6.1.5 TLR9 as a prognostic factor for alimentary tract cancers*

In our series of studies we showed that high TLR9 expression was associated with survival. In the case of oral cancer, TLR9 expression was predictive of poor cancer-specific survival and cancer recurrences in uni- and multivariate analysis (III). In ESCC TLR9 was not associated with survival, even though high TLR9 expression was associated with TNM-class and poor differentiation and increased proliferation (I). This might be due to the limited patient series, only 51 patients were included in the study, and because TNM-class, especially nodal and distal metastases indicate disseminated disease (I). In EAC, however, TLR9 expression was associated with patient survival and metastasis (II).

Sheydihin *et al.* concluded that the lack of TLR9 expression in cancer-associated fibroblasts of esophageal squamous cell carcinoma was a predictor of poor prognosis. (Sheyhidin *et al.* 2011) Other than that, no studies on the effect of TLR9 on gastrointestinal cancer prognosis have been reported.

These results imply that TLR9 might have a use in evaluation of tumors of the alimentary tract, especially in oral carcinoma. TLR9-positive oral (and other) cancers could be treated more intensively with chemo- and/or radiotherapy even when still stage I or without nodal metastasis. However, before TLR9 can be clinically applied as a prognostic factor, larger multi-center series of cancers need to be evaluated to determine the real effects of TLR9 on prognosis.

## 6.2 TLRs in alimentary tract carcinogenesis and cancer progression

### *6.2.1 Bacteria play a key role in TLR signaling in cancer*

The alimentary tract, from the mouth to the anus, is colonized with thousands of species of bacteria, as described in the literature review. The bacteria from the mouth are swallowed through the esophagus to the stomach, where the acidic environment kills most of the bacteria. Sometimes some pathogens, such as *Salmonella* species, but also commensals pass through the stomach to colonize the intestine and colon. Commensal bacteria are crucial for the development of

the immune system and intestinal homeostasis as well as for fighting the pathogenic bacteria. Links between bacterial flora and obesity or cancer have also been described. (Boleij & Tjalsma 2012)

TLRs recognize these bacteria on the basis of their conserved pathogen-associated molecular patterns. Activation of TLRs can either result in production or suppression of immunological reactions, possibly based on the pathogenicity of the bacteria.

In cancer, we and others have shown that TLR9 activation results in induction of a variety of cancerous properties, such as evasion of apoptosis, proliferation and cancer cell invasion (Schmausser *et al.* 2005, Ilvesaro *et al.* 2007, Ren *et al.* 2007, Ilvesaro *et al.* 2008, Ren *et al.* 2009). The opposite mechanisms have been also demonstrated (Rayburn *et al.* 2006, Rayburn *et al.* 2007, Bertin & Pierrefite-Carle 2008, Chiron *et al.* 2009). Why does TLR activation cause different responses in cancerous vs. normal cells? Is it the difference in polarization of TLR-expression or do cancerous cells take advantage of innate immunity receptors? Based on our results, the former seems to be the answer.

In normal oral and esophageal mucosa TLR9 resides in the basal cells, but not in the superficial cells of the epithelium. In gastric and colonic epithelium, a similar basolateral staining pattern has also been observed. We have shown that in epithelial dysplasia TLR9 is expressed strongly in the full thickness of the epithelium. In gastric dysplasia it has also been shown that TLR9 is expressed in the cytoplasm and on the apical side of the cells in addition to the basolateral surface (Schmausser *et al.* 2005). The invasion activity and MMP-induction was similar in cancer cells when we used DNA of highly pathogenic *H. pylori* and less pathogenic *E. coli*, which is actually a part of our normal bacterial flora. This suggests that it is not simply the presence of bacteria, but rather their specific location that causes a normal immune response or promotion of cancerous properties.

In cancerous and premalignant transformation one of the main histological properties is to define whether the cells are polarized or not. Possibly when cellular polarization is lost, the polarization of TLRs is also lost, resulting in abnormal activation of, for example, TLR9 by commensal bacteria, which normally do not invade the mucosa. In cancer and dysplasia, the tight junctions and other cell-to-cell-contacts are also disturbed, which allows bacteria to invade the intercellular space and cause TLR activation in the deeper layers of squamous epithelium.

Taken together, there appear to be multiple steps involving bacteria and the immune system in the carcinogenesis of alimentary tract. These steps include at least 1) changes in local bacterial flora, 2) loss of polarity in the epithelial cells, 3) increased permeability of the intestinal wall due to loosened junctions, 4) invasion of bacteria through the epithelium, 5) recognition of bacterial components by innate immunity receptors of the epithelial and immune cells and 6) cancer cell invasion, proliferation and evasion of apoptosis. (Figure 2.)

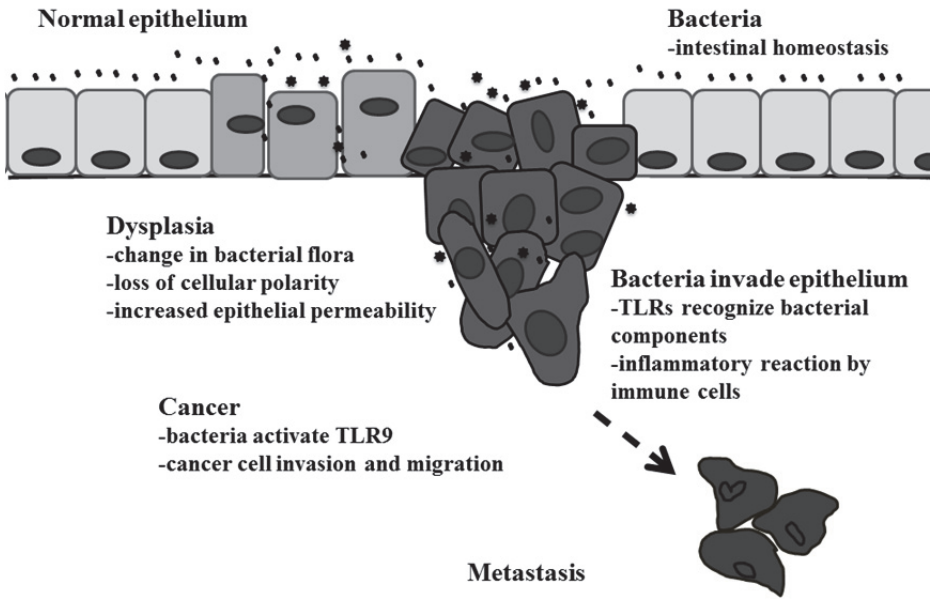


**Fig. 2. The proposed role of bacteria in alimentary tract cancer progression. The commensal bacteria colonize the intestine, maintaining homeostasis. In dysplasia, the epithelial cells lose their polarity and bacteria invade the epithelium, activating TLRs. The activation of TLRs cause cancer cells to invade and migrate, causing metastasis.**

### *6.2.2 Endogenous DNA released by cancer cells could activate metastasis*

Cancerous cells undergo apoptosis more frequently than normal cells. In our study we could activate cancer cell invasion with a G-quadruplex and a 9-mer DNA oligonucleotides. Both of these oligonucleotides had a phosphodiester-

backbone, which resembles normal DNA and is not endonuclease resistant. In vivo this would mean that as the cancer cells die, they release DNA for other cancer cells to use for invasion. Also cancer cells killed by chemotherapy could be utilized by the living cancer cells for metastasis purposes. Such effects have indeed been recently demonstrated *in vitro* by Tuomela *et al.* (Tuomela *et al.* 2013a).

This is possible due to the extraordinarily fast proliferation rate, which results in tumor growth. At the same time, DNA from dying cells is released to the intercellular space via apoptosis and necrosis. indeed, an increased amount of DNA has been detected from the blood of patients receiving chemotherapy. (Deligezer *et al.* 2008a, Deligezer *et al.* 2008b)

## 6.3 Shortcomings in materials and methodology

We assessed the expression and function of TLR9 in seven different cell lines, but we had only one cell line from each cancer type, except in oral cancer where three cell lines were investigated. This can be considered as a shortcoming of our study as the cell lines used might not represent the average cancer of that type. The results obtained, however, speak against this argument, as the behavior of all the cell lines was very similar when treated with CpG-ODNs. The matrix metalloproteinase expression pattern differed between the cell types. Had we used more than one cell line from each cancer, we could have determined whether the differences in MMP-mRNA induction were dependent on the cell line or the cancer type.

We used series of patients from different gastrointestinal malignancies to determine the clinicopathologic associations with TLR9 and the prognostic role of TLR9 in these cancers. In study I the number of the patients (51) was too small to reliably determine whether TLR9 has any effect on esophageal squamous cell carcinoma prognosis. Results by Sheyhidin *et al.* suggested that the expression of TLR9 in fibroblast-like cells in esophageal squamous cell carcinoma was associated with better outcome. They found no association between TLR9 expression in tumor cells and survival in their 87 patients. (Sheyhidin *et al.* 2011)

In esophageal adenocarcinoma, no other studies of TLR9 had been published at the time of writing. In oral squamous cell carcinoma no associations between survival and TLR9 expression have been shown. Kotrashetti *et al.* have studied TLR9 expression in a small patient cohort of 27 patients with oral squamous cell carcinoma. (Kotrashetti *et al.* 2013)

In study IV the analysis of MMP-induction was based on mRNA-data. However, mRNA data does not always accurately reflect expression levels as alternative splicing and ribosomal activation also effect protein production. Thus, more reliable results could have been obtained by measuring protein levels rather than mRNA.

In this study we also did not confirm the TLR9-dependency of CpG-ODN stimulation. This could have been done by using siRNAs or TLR9-antibodies. According to other studies by us and others the TLR9-dependency of CpG-ODN-mediated invasion has been confirmed in numerous other cancers (Tables 3–6). The downstream pathway from TLR9 to MMP-induction has also been characterized before in breast cancer. (Merrell *et al.* 2006, Ilvesaro *et al.* 2007, Ilvesaro *et al.* 2008)

## 6.4 Clinical implication of TLR9 in alimentary tract cancers

In alimentary tract cancers, upregulation of TLR9 was shown to be highly prevalent. TLR9 is highly expressed in squamous cell dysplasia (I). This finding suggests that the immunohistochemistry of TLR9 could be applied for esophageal squamous dysplasia detection. TLR9 evaluation may also be used for evaluation of oral and esophageal cancer prognosis and for choosing the appropriate treatment, e.g. whether to progress with extensive lymph node dissection and chemo- or radiotherapy or to apply normal surgical protocols (II, IV, Sheydihin *et al.* 2011). However, the studies need to be repeated in larger patient cohorts before clinical approval of this test.

TLR9 is a promising prognostic factor, but also a possible therapeutic opportunity. TLR9 agonists, CpG-oligodeoxynucleotides, have been tested in various cancers for their immune system-activating properties. (Jahrsdorfer & Weiner 2008) TLR9 agonists in cancers are used because CpG-ODNs activate TLR9 in the immune cells. Activation of the immune system produces an anti-cancer response in some cancer types (Nierkens *et al.* 2011). Much less studied is the TLR9 antagonist chloroquine, which is already in use as an antimalarial drug and in treatment of autoimmune diseases, such as rheumatoid arthritis (Stohl & Arkfeld 1996, Baird 2005, Tuomela *et al.* 2013b). Based on our studies, chloroquine could be potentially used as an adjuvant treatment for alimentary tract cancers to prevent cancer invasion. Chloroquine has been shown to be effective in studies in a murine model of triple-negative breast carcinoma, but

according to our results, chloroquine did not affect cancer progression in a mouse xenograft model of breast cancer. (Jiang *et al.* 2010, Loehberg *et al.* 2012, Tuomela *et al.* 2013b) This effect could be explained by the dosing of chloroquine in the study, or the cell lines used. (Tuomela *et al.* 2013b)

In addition to the role of *H. pylori* in gastric carcinoma, it has also been shown that bacterial flora undergoes changes in colorectal cancer and in Barrett's esophagus. (Chang *et al.* 2006, Yang *et al.* 2009) In BE, which is a prerequisite for esophageal adenocarcinoma, the bacterial flora was more anaerobic and gram-negative when compared to normal esophagus. (Yang *et al.* 2009) These observations combined with our results imply that bacteria could affect the carcinogenesis and cancer progression in alimentary tract cancers. This information could be used clinically to alter the bacterial flora of individuals, based on their bacterial composition. This could be done by using antibiotics or probiotics, which support the growth of other species. Also fecal transplantation used to treat, for example, C. difficile infection, could be tested as an adjuvant- or preventive treatment for cancer. (Mattila *et al.* 2012) The most effective way to affect bacterial flora would be by exposing children to different bacterial species, but this kind of preventive treatment would have many ethical issues, since the other effects of changing one's bacterial flora are not known, especially in childhood.

# 7 Summary and conclusions

In the present study we confirmed that TLR9 is expressed in cancers of the alimentary tract. Furthermore, we confirmed that TLR9-agonists induce invasion in cancer cell lines derived from the gastrointestinal area. More specifically the conclusions were:

1. TLR9 is expressed in normal lingual and esophageal epithelium.
2. The expression of TLR9 is upregulated in esophageal squamous dysplasia and thus might be a useful diagnostic aid.
3. TLR9 is expressed and functional in various alimentary tract cancers, including oral squamous cell carcinoma, and might be useful as a prognostic factor in EAC as well as OTSCC. TLR9 mediates oral squamous cancer cell invasion and migration in vitro. TLR9 may represent a pathogenetic link between bacteria and oral cancer.
4. The natural endogenous and bacterial DNA-ligands in the phosphodiester backbone, as well as the synthetic ligands in the phosphothioate backbone can activate cancer cell invasion in vitro. This effect is mediated by matrix metalloproteinases and tissue-inhibitor of metalloproteinase-3 in esophageal adenocarcinoma, gastric adenocarcinoma and colorectal adenocarcinoma in a cell line-dependent manner.

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I Takala H, Kauppila JH, Soini Y, Selander KS, Vuopala KS, Lehenkari PP, Saarnio J & Karttunen TJ (2011) Toll-like receptor 9 is a novel biomarker for esophageal squamous cell dysplasia and squamous cell carcinoma progression. J Innate Immun 3(6): 631–638.

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