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Microtubule and Cdc42 are the Main Targets of Docetaxel's Suppression of Invasiveness of Head and Neck Cancer Cells

Yasunao Kogashiwa[1], Hiroyuki Sakurai[2] and Naoyuki Kohno[1]
[1]*Department of Otorhinolaryngology, Head and Neck Surgery, Kyorin University School of Medicine,*
[2]*Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Japan*

1. Introduction

Many squamous cell carcinoma (SCC) of the head and neck presents with locally advanced disiease. In such cases, a combination chemotherapy of docetaxel, cisplatin, and 5FU, followed by radiation improved their survival (Posner et al. 2007; Vermorken et al. 2007). That is, addition of docetaxel to the combination of cisplatin and fluorouracil improves survival in head and neck squamous cell carcinoma. In a recent work to elucidate the possible mechanism, we investigated the effect of docetaxel on cell movement using head and neck cancer cell lines Hep2 and Ca9-22. Docetaxel treatment suppressed migration and invasiveness of head and neck cancer cells in vitro. We investigated the downstream effectors that control invasiveness after docetaxel administration in the present work.

2. IC_{10} and IC_{50} in HEp-2 and Ca9-22 cells

We used the same IC_{10} and IC_{50} concentrations (Table 1) (Kogashiwa et al. 2010) as our previous study. At IC_{10} concentrations, anti-proliferative effect was not observed.

Cell line		IC_{10}	IC_{50}
HEp-2	Cisplatin	2 μM	20 μM
	Docetaxel	5 nM	23 nM
Ca9-22	Cisplatin	10 μM	40 μM
	Docetaxel	6 nM	11 nM

Table 1 IC_{10} or IC_{50} values in two cells
IC_{10} or IC_{50} values after 1 hour drug exposure followed by a 96 hours incubation in two head and neck cell lines.

3. Docetaxel inhibits the migration of head and neck cancer cells

To assess cell migration a wound healing assay was employed. These results have been reported (Kogashiwa et al. 2010). Briefly, both in HEp-2 cell and CA9-22 cell, wound closure relative to no treatment condition is significantly reduced in docetaxel treatment while cisplatin treatment does not affect the cell migration (Fig.1.) (Kogashiwa et al. 2010).

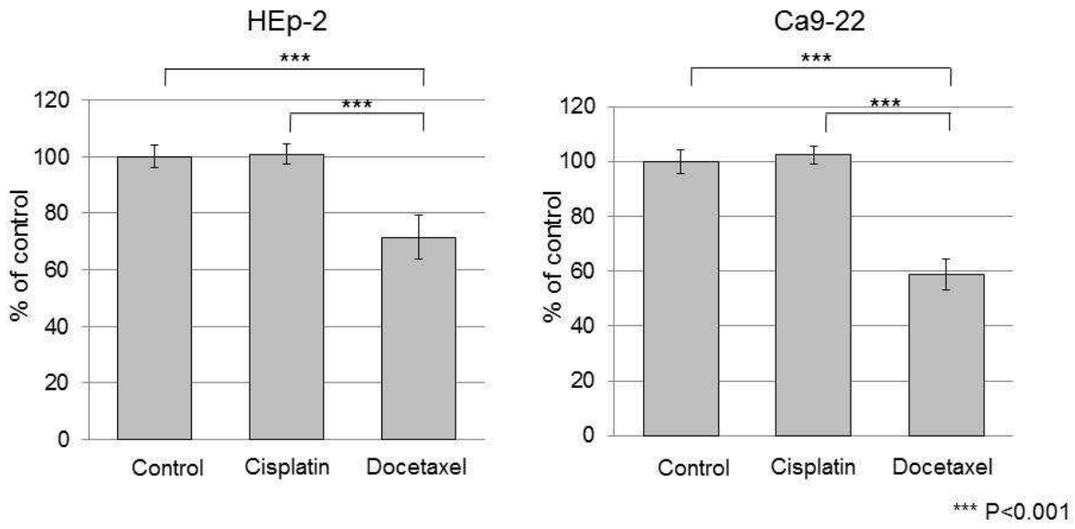

Fig. 1. Migration assay at IC10 in two head and neck cancer cell lines.
Migration rate is compared to control cell migration rate. Each data point represents mean ± SE. ***p < 0.001. 15 replicate were used in each experiments and experiments were repeated 4 times.

4. Docetaxel inhibits the invasiveness of multicellular tumor spheroids.

The similar results are obtained in three-dimensional multicellular tumor spheroid culture (Kogashiwa et al. 2010). At IC_{10} determined in monolayer culture, either cisplatin or docetaxel does not affect filopodia formation. However, at IC_{50} determined in monolayer culture, docetaxel, but not cisplatin, significantly decreases filopodia formation in HEp-2 cells in spheroid culture (Fig.2.) (Kogashiwa et al. 2010). Taken together, In the previous study, we have shown that docetaxel, but not cisplatin inhibits cell migration both in 2D and 3D culture.

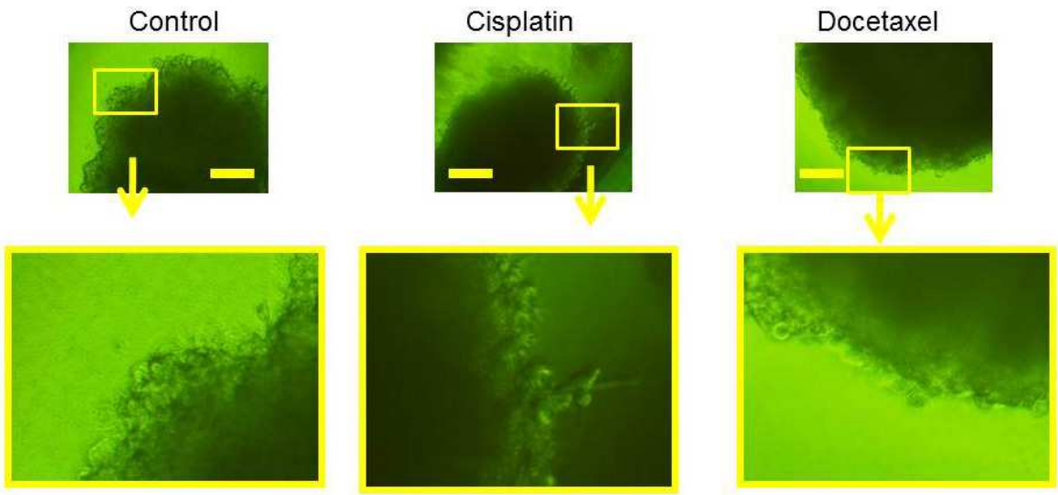

Fig. 2. 3D gel culture of HEp-2 cell spheroids at IC50 concentration.
HEp-2 cell spheroids were treated with cisplatin or docetaxel at IC50 followed by 96-hour incubation. bars= 250 μm. 4 replicate were used in each experiments and experiments were repeated 4 times.

5. Tubulin bundle was formed by docetaxel treatment

Taxanes, including docetaxel, function as a mitotic spindle toxin by inhibiting microtubule turnover. They bind to microtubules and enhance tubulin polymerization. We hypothesized that docetaxel may exert similar effect on cytosolic, non-centrisome associated microtubules resulting in decreased cell motility. We therefore examined the structure of microtubules as well as actin filaments. Consistent with previous observation, filopodia formation was less in docetaxel treatment compared to cisplatin treatment. But no gross abnormality was found in actin filament structure between the treatments. On the other hand, tubulin bundle formation was noted in docetaxel treatment but not in cisplatin treatment (Fig.3.). Then, we attempted to find the mechanism that connect deformed microtubule and decreased filopodia formation.

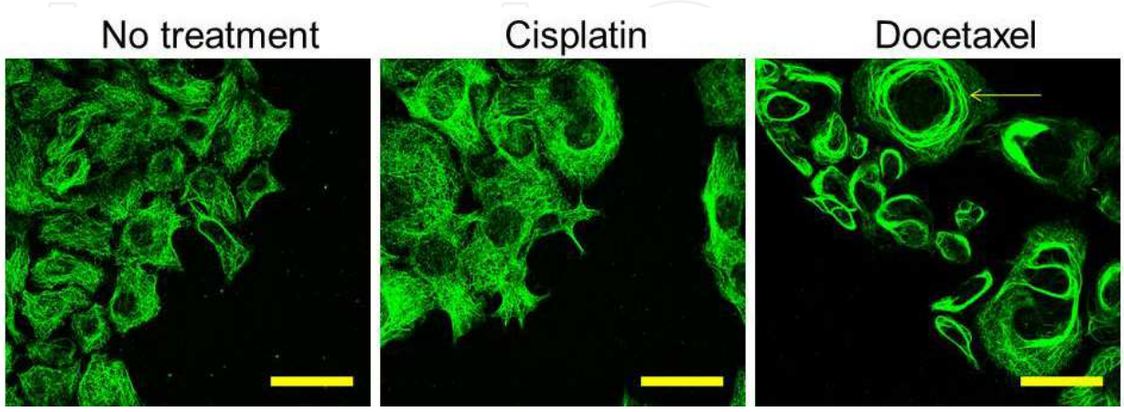

Fig. 3. Staining of α-tubulin (by antibody) in HEp-2 cells treated by cisplatin, docetaxel or no treatment at IC50. Bars=50 μm. 3 replicate were used in each experiments and experiments were repeated 4 times.

6. Docetaxel inhibited Cdc42 activity

Rho GTPases regulate many essential cellular processes, including actin dynamics, gene transcription, cell-cycle progression, cell adhesion, tumor progression and invasiveness (Hall 1998; Schmitz et al. 2000; Price et al. 2001). Among Rho-GTPases, Cdc42 is previously implicated in connecting microtubular input to actin filament organization (Cau et al. 2005). Cdc42 also promotes leading-edge extension through activation of Rac, which is implicated in formation of lamellipodia (Bishop et al. 2000). Thus, we examined activity of Cdc42, Rac and RhoA in the cells underwent cisplatin or docetaxel treatment, or no treatment. At IC_{10} concentration, docetaxel significantly decreased Cdc42 and Rac activity in HEp-2 cells, but not RhoA activity (Fig.4.; cited from (Kogashiwa et al. 2010) with modification). Total amount of Cdc42, Rac and RhoA was not significantly different among these three conditions.

It is reported that Cdc42 is activated in a thin band at cell edges extending filopodia (Nalbant et al. 2004). Consistent with the results of activity assay, Cdc42 localized at the plasma membrane was decreased after docetaxel treatment at IC_{10}. Localization of Rac1 and RhoA had no apparent changes after treatment compared to control.

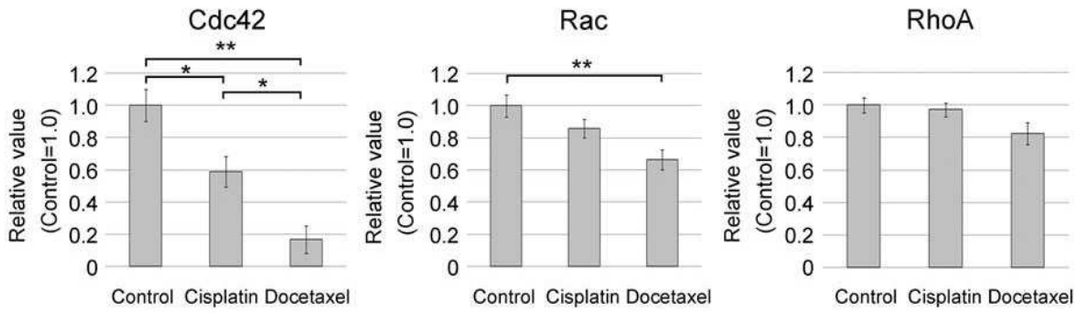

Fig. 4. Colorimetric assay of Cdc42, Rac and RhoA activity in HEp-2 cells.
The levels of activated Cdc42, Rac and RhoA in HEp-2 cell were evaluated immediately after 1 hr of indicated treatment. Each data point represents mean ± SE. *$p < 0.05$, **$p < 0.01$.

7. The molecules implicated in actin cytoskelton regulation were not significantly different between cisplatin and docetaxel treatment.

Lamellipodia or filopodia formation was suppressed when cells were treated with docetaxel (fig.5.). Ezrin/radixin/moesin (ERM) proteins link the cortical cytoskeleton to the plasma membrane. In their active conformation (i.e. phosphorylated ERM), the N-terminal ERM domain binds to the cytoplasmic tails of transmembrane proteins, and the C-terminal ERM association domain binds to actin filaments. Using a p-ERM antibody, the levels of active ERM proteins were evaluated by Western blotting after no treatment, cisplatin or docetaxel treatment at IC_{10}. There was no significant difference in the level of p-ERM among cisplatin, docetaxel and no treatment over a time course up to 48 hours (fig.6.). We also investigated the cofilin pathway as a regulator of the actin cytoskeleton. Cofilin is able to bind both G-actin and F-actin, and regulated by LIM kinase 1 and its related kinases. The levels of cofilin (fig.6.) and LIMK1 (fig.6.) were evaluated over a time course of treatment at IC_{10}. The levels of these proteins were not significantly different among cisplatin, docetaxel and no

treatment, either. These results suggest that docetaxel treatment does not directly affect actin cytoskeleton remodeling.

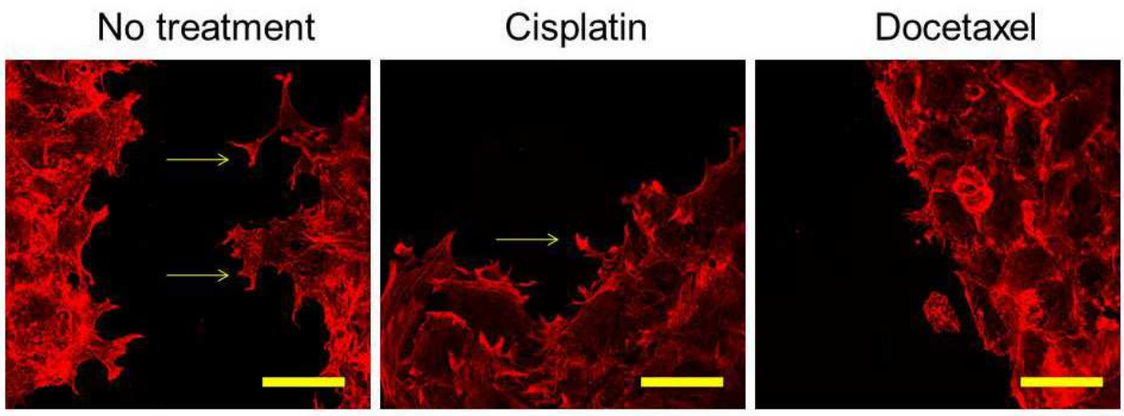

Fig. 5. Staining of F-actin (phalloidin) in HEp-2 cells treated by cisplatin, docetaxel or no treatment at IC50. Bars=50 μm. 3 replicate were used in each experiments and experiments were repeated 4 times. Arrows; filopodia

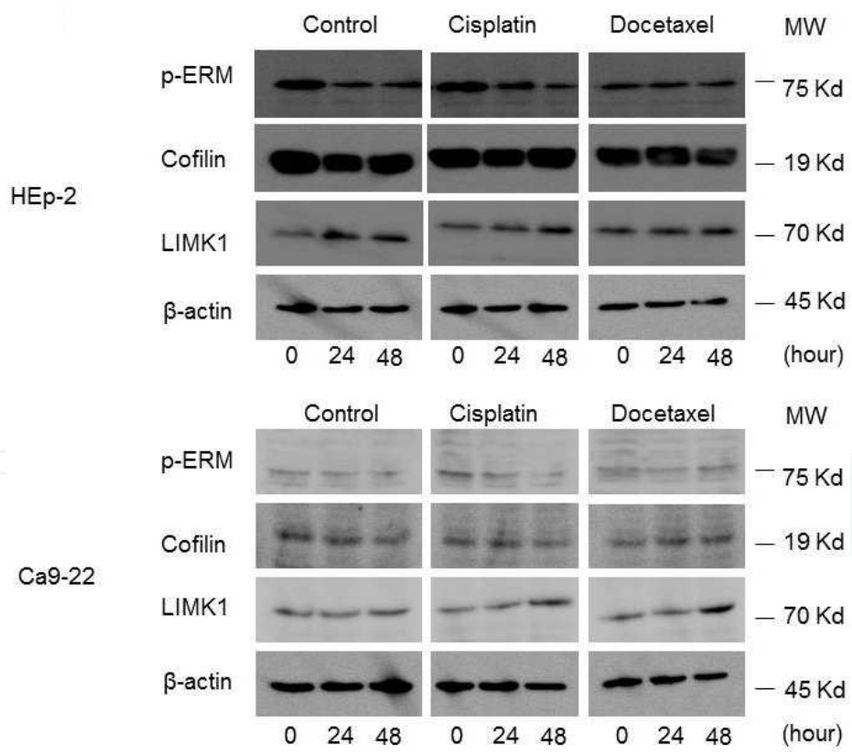

Fig. 6. Time course of the levels of p-ERM, cofilin and LIMK in HEp-2 cells and Ca9-22 cells treated with cisplatin or docetaxel.
β-actin was probed for loding control. 3 replicate were used in each experiments and experiments were repeated 3 times.

8. Docetaxel treatment did not promote epithelial-mesenchymal transition (EMT)

It has been well documented that many cancer cells lose most of their epithelial characteristics during progression and metastasis, through the process of EMT (Thiery 2002). Generally, EMT causes increased motility and invasiveness of cancer cells due to decreased cell-cell adhesion. Snail, a zinc finger transcription factor, triggers EMT through direct repression of E-cadherin transcription (Batlle et al. 2000; Cano et al. 2000). The reverse correlation of snail and E-cadherin expression has been reported for various human cancers, including SCC (Yokoyama et al. 2001). Accordingly, we investigated the snail and E-cadherin expression levels to assess whether cisplatin and/or docetaxel at IC_{10} differently influences EMT. Snail was decreasing over a time course (Fig.7.). Conversely E-cadherin was increasing over a time course (Fig.7.). But the levels of these proteins were not significantly different between cisplatin, docetaxel and no treatment. These results indicate that docetaxel treatment does not promote EMT at least in these cell lines.

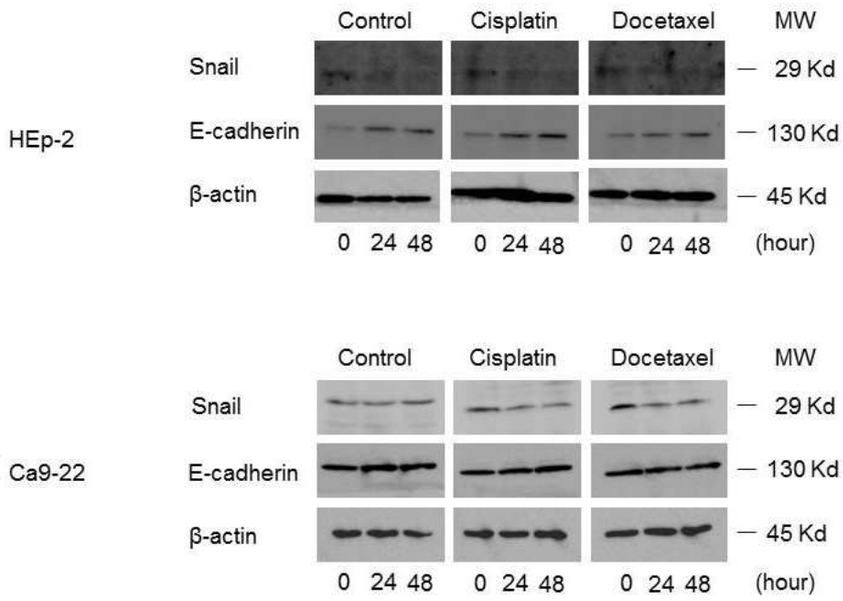

Fig. 7. Time course of snail and E-cadherin expression of HEp-2 cells and Ca9-22 cells treated with IC_{10} concentration of cisplatin or docetaxel.
β-actin was probed for loding control. 3 replicate were used in each experiments and experiments were repeated 3 times.

9. Matrix metalloproteinase (MMP) production was not significantly affected by cisplatin and docetaxel treatment

MMPs are known to play an important role in extracellular matrix remodeling during the process of tumor invasion and metastasis (Egeblad et al. 2002). Two of these enzymes, MMP-2 and MMP-9, are potent gelatinases and have been correlated with the processes of invasion and metastasis of SCC (Sheu et al. 2003; Patel et al. 2005). Gelatin zymography revealed prominent 72000 dalton bands, corresponding to MMP2 secreted from the HEp-2 and Ca9-22 cells. These bands appeared unchanged by either cisplatin or docetaxel treatment (Figure 8).

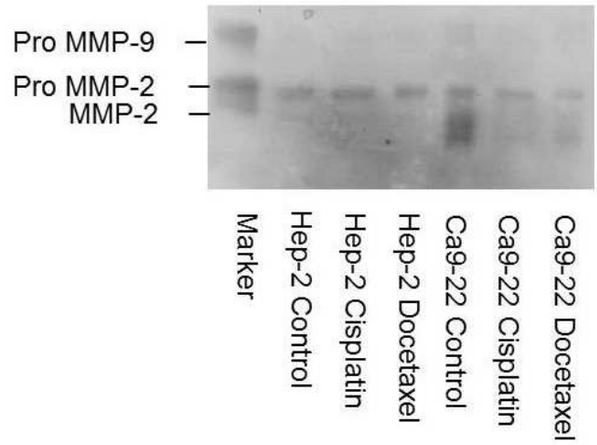

Fig. 8. Gelatin zaymograpy.
MMP secretion in HEp-2 cells and Ca9-22 cells after treatment or control were evaluated by Gelatin zymography. Gelatin zymography revealed prominent 72000 dalton bands, corresponding to MMP2 secreted from the HEp-2 and Ca9-22 cells. These bands appeared unchanged by either cisplatin or docetaxel treatment

10. Discussion

The metastatic process has traditionally been viewed as follows: (1) detachment of individual cells from the primary lesion (2) invasion of local stroma (3) entry of single cells or aggregates of tumor cells into blood vessels directly or via lymphatic channels (intravasation) (4) sticking to the vasculature distant from their origin followed by extravasation, and (5) invasion into the parenchyma of the target organ site. The newly formed lesions can themselves become the source of disseminating cells which repeat this cycle, giving rise to tertiary metastasis. Thus, Inhibition of invasion in the primary lesion

should result in preventing the distant metastasis. From this point of view, our results suggest that docetaxel, which decreased local invasiveness, may prevent distant metastasis. Although the effect of docetaxel on cell migration or invasiveness of ovary cancer cells (Bijman et al. 2008) and umbilical vein endothelial cells (Bijman et al. 2006) have been described, its effect on head and neck cancer cells has not been evaluated.

Actin cytoskeleton provides the driving force for cell migration, while microtubules are required to establish cell polarity during motility in fibroblasts (Bershadsky et al. 1991). Actin is regulated by Rho family small GTPases, and it is indicated that microtubules may influence actin cytoskeleton through modulation of the activity of Rho GTPases (Wittmann et al. 2001). Among Rho GTPases, cdc42 was reported to control the polarity of actin and microtubule through distinct signal transduction pathways (Cau et al. 2005). It is possible that the abnormal tubulin bundle induced by docetaxel lead to suppression of cdc42 activity. This decreased cdc42 activity could affect actin filament and decrease the migration of the head and neck cancer cells.

In contrast, we could not find definitive evidence for docetaxel to directly affect actin cytoskeleton regulation. It did not affect EMT processes or MMP production of these head and neck cancer cell lines.

11. Conclusion

In conclusion, it is likely that docetaxel suppresses SCC migration through inhibition of microtubule turnover, which affects cdc42 activity and its subcellular localization leading to decreased filopodia formation. We propose that effect on cancer cell migration should be assessed together with anti-proliferative activity when evaluating a cancer chemotherapeutic agent. Along this line, we are now evaluating anti-migratory effect of EGFR tyrosine kinase inhibitors, another class of promising treatment for head and neck cancer.

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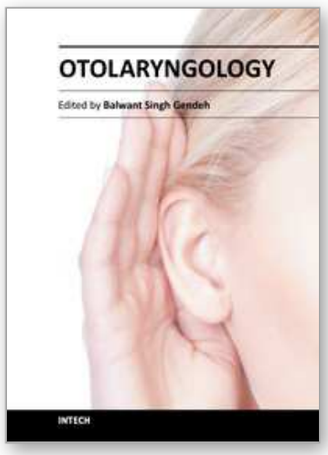

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This book emphasizes on different aspects of otolaryngology - the medical sciences of diagnosis and treatment of ENT disorders. "Otolaryngology" is divided into various clinical sub-specialities, namely otology, rhinology, laryngology, and head and neck. This book incorporates new developments, as well as future perspectives in otolaryngology. I would like to dedicate this book to those of you who will pick up the torch and by continued research, close clinical observation and the highest quality of clinical care, as well as by publication and selfless teaching, further advance knowledge in otolaryngology from this point forward. It is intended to be a guide to other books to follow. Otolaryngologists, researches, specialists, trainees, and general practitioners with interest in otolaryngology will find this book interesting and useful.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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